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IMPROVEMENT OF REPRODUCTIBILITY AND QUALITY CONTROL OF HUMAN GROWTH HORMONE RADIOIODINATION

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SUMMARY: The labelling reaction of human growth hormone (hGH) with ^{125}I and its chromatographic purification have been studied with emphasis on the reproducibility of the yields, quantitative recoveries and resulting activities. Through the accurate standardization of a monitoring technique, it is confirmed that there are no significant losses in radioactivity or protein during the labelling or purification process. By strict control of the reaction conditions, a fairly good reproducibility is also obtained in the labelling of various hGH extracts with different ^{125}I shipments used after short or long storage. Finally, the specific activity (or absolute mass) of the radioiodinated protein is determined by this Analysis of the Reaction Mixture and compared to the widely used radioimmunological assay (Self-displacement). The agreement between the two methods can be considered satisfactory. In order to express the specific activity in microcuries per microgram, a simple coincidence method for determining the absolute counting rate of ^{125}I has been adapted to our facilities. This type of purification and quality control can be applied to any radioiodinated protein and allows one to obtain reproducible tracers, which is indispensable for the improvement of between-assay and between-laboratory precision.

KEY WORDS: **Radioiodination. Human Growth Hormone (hGH). ^{125}I. Radioimmunoassay. Specific Activity.**

INTRODUCTION

Protein radioiodination is considered to be a difficult process to control, being highly variable the yields and specific activities and even the labelling itself[11, 12, 13, 15, 23, 24]. Moreover, accurate determination of the mass of labelled antigen is in itself quite difficult, due to the extremely low concentrations present and interference from ill-defined "preparation damage"[8, 11, 14, 27, 30, 32, 37 40]. For these reasons the specific activity of a labelled protein is seldom stated in commercial kits or even in published works, where at best approximate values are reported. Though such approximations are sometimes still compatible with the establishment of radioligand assays for clinical use, a better standardization and understanding of the equilibria involved requires that one knows with reasonable certainty the mass of reagent one is dealing with[25]. The use of better standardized and controlled tracers is extremely important not only for improving the intra- and inter-laboratory reproducibility of these assays[16, 26], but also in all tracer studies involving immunological, receptor or other biological mechanisms where the reagent concentration and chemical nature plays a fundamental role.

For a tracer specific activity determination, one can utilize either of two well-established techniques. The first, called "analysis of the reaction mixture"[15 10] or "column recovery"[17], is based primarily on the original Chloramine T labelling work[19]. Although it required only a simple separation step, the overall recovery of tracer (^{131}I-hGH in that case) was only about 35%. Moreover, the short Sephadex G-50 column used did not discriminate high molecular weight species such as "damaged components". Hence, one cannot take into consideration the possible presence of aggregate, of ^{125}I carried by serum albumin or of other undesirable effects[4]. Such a purely radiochemical method does not take into account any possible loss in immunoactivity of the tracer. The second method is the so-called "self-displacement" or "autodisplacement"[9, 15, 17, 18, 20, 29, 33, 35, 36] and is purely radioimmunological. It has the characteristic of determining a specific activity based only on immunoactive molecules, but introduces new variables and uncertainties that can affect the final result: comparison with a reference preparation, possible variation in antibody titre and avidity between different assays, possible difference in purity, behaviour and immunoactivity between the labelled and unlabe-

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lled antigen, deterioration of the latter, etc. A third method, defined as "isotopic dilution", has also been described[17]; since it is based on radioimmunological binding, this method shares the same limitations mentioned above.

The goal of the present work was to standardize labelling, purification and monitoring conditions. For this purpose we introduced a technique of analysis of the reaction mixture capable of controlling reproducibility and quantitative recoveries. Finally, we performed a comparison with the self-displacement technique on various preparations labelled at different specific activities. In the process we took advantage of a polyacrylamide gel electrophoresis (PAGE) technique for the "clean" B/F separation[1], detecting and identifying, as previously described[2, 4], the undesirable components present after labelling and purification.

To determine counter efficiency, the coincidence method described by Horrocks[21, 22], adapted in this laboratory to a common single channel well-type NaI (T1) detector, was used. Thus, by direct determination of the absolute radioactivity of ^{125}I, the error that can be introduced by the simple use of ^{129}I[34], which does not have the sum peak, was avoided. In this manner it was possible to control the amount of radioisotope actually employed and to express the specific radioactivity in real terms of microcuries per microgram of protein, facilitating inter-laboratory comparison.

MATERIALS AND METHODS

Human growth hormone (HS 2243-E), immunoassay grade, was kindly provided by the National Institute of Arthritis, Diabetes, Digestive and Kidney Diseases (NIADDK, Baltimore, USA); hGH-IPEN L-8, 9, 14 and 16 were different lots, all extracted in this laboratory according to the method of Roos et al.[31]; guinea pig antiserum against hGH was also obtained from NIADDK, already at a dilution of 1:2000.

$Na^{125}I$, with specific activities around 400-500 μCi/μl, was purchased from New England Nuclear (Boston MA, USA).

Sephadex G-100 (4-120 μ) was obtained from Pharmacia (Uppsala, Sweden) and bovine serum albumin (BSA), fraction V, from Sigma Chemical Co. (St. Louis MO, USA). The counter was a Nuclear Chicago 1185 series, 300 sample, single-channel well-type Automatic Gamma Counting System. In some of the later experiments, a Beckman Gamma 4000 Counting System (200 samples), which had an almost identical efficiency for ^{25}I, was used.

Polyacrylamide gel electrophoresis (PAGE) was carried out as previously described[1].

Coincidence method for the determination of the absolute counting rate of ^{125}I samples.

The establishment of a fast, simple, sufficiently sensitive and accurate technique for determining the activity of ^{125}I became necessary in our laboratory. Ion chamber detectors did not exhibit sufficient sensitivity and precision in the case of low activity ^{125}I samples and ^{125}I calibration standards for the gamma counter were expensive and unavailable to us. The method we used was basically that described by Horrocks[21], adapted to our counter, which was calibrated with the ^{129}I 40 KeV peak to have the full scale range set at approximately 100 KeV. Sweeping the window in 10% steps (10 KeV each) an energy spectrum of ^{125}I was obtained. The good resolution of the two main peaks (single = N_s and coincidence = N_c) is shown in Fig. 1. The absolute counting rate (S) and consequently the counter efficiency, were calculated through the formula: $S = (N_s + 2N_c)^2/(4N_c)$. The precision of the method, which requires less than half an hour to perform, is shown in Table 1.

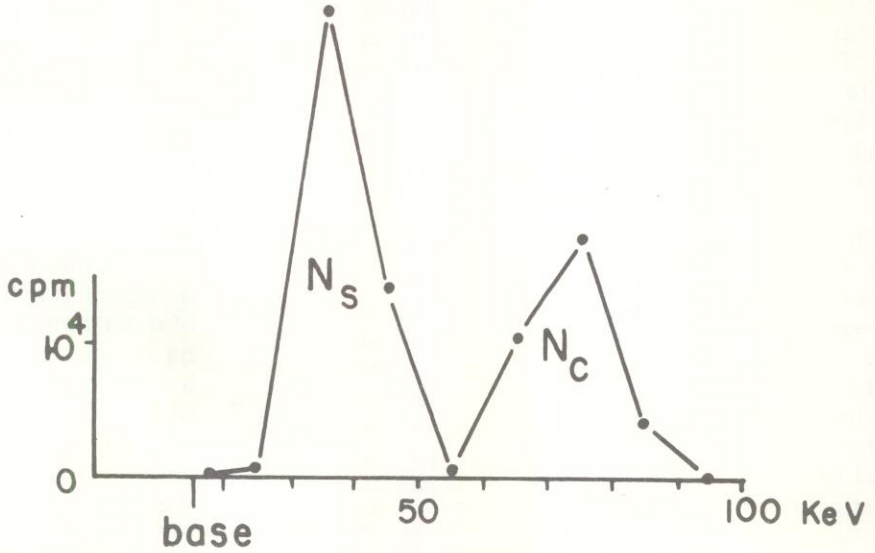

Fig. 1 — Gamma spectrometry of $Na^{125}I$, using the single channel, well-type Nuclear Chicago gamma counting system.
N_c = number of counts in coincidence N_s = number of single counts.

TABLE 1
Determination of the absolute counting rate of ^{125}I samples

$Na^{125}I$ monthly shipment	Stated Activity*1 (μCi/μl)	Activity Found*2 (μCi/μl)	Percent Difference	Calculated Counter Efficiency(%)
1	380.0	408.7	+7.6	82.3
2	442.5	429.2	−2.3	81.7
3	445.0	421.6	−5.3	81.0
4	387.0	396.0	+2.1	81.0
5	315.7	330.2	+4.6	81.1
6	429.9	446.7	+3.9	81.2
7	447.0	459.9	+2.9	80.0
			$\bar{x}$ =	81.1
			SD = ±	0.7
			CV =	0.87%

*1 Corrected for the decay
*2 Each value is the average of 4 contings, two each for separate 1 μl aliquots withdrawn directly from the shipping vial and appropriately diluted (Hamilton syringe)

Standardized labelling and counting for the specific activity determination via Analysis of the Reaction Mixture.

The labelling was carried out with the Chloramine T method of Greenwood et al.[19]. Protein concentrations for IPEN and NIADDK-hGK preparations were determined by the method of Lowry et al.[28]. The reagents, all freshly prepared, were added under continuos magnetic stirring in the following order: 40 μl sodium phosphate buffer 0.5 M, pH 7.4; 0.5-3 μl $Na^{125}I$ in NaOH 0.1 M; 5 μl hGH (1 mg/ml); 10 μl Chloramine T (5 mg/ml) and, after 30 seconds, 20 μl sodium metabisulfite (10 mg/ml). The last three reagents were freshly dissolved in sodium phosphate buffer (0.05 M, pH 7.4) just prior to the addition. Once the labelling reaction was completed, Veronal buffer (0.025 M, pH 8.6) containing 1% BSA and carrier NaI was added (200 μl). After vigorous stirring, 10 μl aliquots of this solution were withdrawn, appropriately diluted and counted. The tube and stirrer used in the labelling and the tip of the pipette used to apply the labelling mixture to the top of the Sephadex G-100 column for purification were also counted. By adding known amounts of radioactivity, it was verified that the error due to geometry and self-adsorption was negligible in these experiments. The radioactivity adsorbed to these surfaces in fact seldom exceeds 1% of the total used in the labelling. The values obtained through this monitoring were used to calculate the total amount of ^{125}I used in the reaction which, after correction for the counter efficiency, could be expressed in microcuries. The total amount of radioactivity contained in each tube following elution from Sephadex (Fig. 2) was evaluated using the same geometry and technique. In all cases, 10 μl aliquots, taken with the same micropipette were counted in conical aluminum foil. This type of container serves in our laboratory as a cheap, discardable, non-gamma absorbing system. Indeed, it was found that glass and some type of plastic containers can reduce the counter efficiency for ^{125}I by up to 20%. Comparison between the total radioactivity added to the column and the total recovered in the tubes, provided the overall recovery data shown in Table 2. The fact that very little, if any, radioactivity is lost on the Sephadex columm, as implied by the high recoveries, was confirmed by unpacking the column following chromatography and resuspending and counting an aliquot of Sephadex gel; less than 1% of the total radioactivity was found to be present in the column.

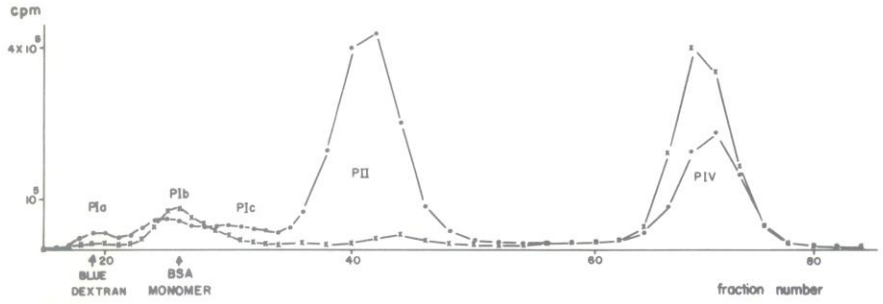

Fig. 2 — Typical Sephadex G-100 purification of radiolabelled hGH. Colum size, 2x45 cm; flow rate, 12 ml/h; fraction volume 2.0 ml. — • — hGH; — x — BSA. PIa, aggregate form; PIb, BSA-related radioactivity; PIc, "big" hGH; PII, ^{125}I-hGH; PIV, free radioiodide.

TABLE 2
Specific activities calculated according to the Analysis of the Reaction Mixture

Exp. N°	hGH lot N° and source	$Na^{125}I$ age (days)	μCi of ^{125}I used in the labelling	Total Recovery (%)	PII (%)	Free ^{125}I (%)	Specif. Activ. (μCi/μg)
1	IPEN L-8	40	858	86.2	51.6	28.9	88.5
2	"	33	794	85.6	58.7	20.1	100.0
3	"	25	913	102.7	51.0	28.2	117.4
4	"	8	930	85.8	51.9	29.7	114.9
5	"	16	694	106.2	57.8	24.3	94.5
6	HS-2243E	38	755	98.8	54.7	28.9	93.9
7	"	29	748	98.5	56.4	24.2	98.6
8	IPEN L-9	27	749	93.4	57.2	25.4	91.2
9	IPEN L-14	25	854	101.8	57.3	21.3	108.6
10	IPEN L-16	21	846	93.1	58.2	24.8	110.5
				$\bar{x}$ = 95.2	55.5		101.8
				SD = ± 7.6	± 3.0		± 10.3
				CV = 7.9%	5.3%		10.1%

Specific activity determination via the Self-displacement method.

The method employs two radioimmunoassay curves, one having "x" fixed amounts of labelled hGH (always taken from PII, Fig. 2) and increasing quantities of cold hormone (the same used in the labelling) and the other having increasing amounts of labelled hormone alone. The B/F values relative to each x, 2x, 3x, etc., increase in labelled hormone forming the

second curve can be converted to picogram of hormone on the first curve, providing an average value of the specific activity in cpm per picogram (Fig. 3). In most of the experiments reported, we employed B/F separation on PAGE as in previous radioimmunoassay work[1], utilizing 20-30 pg of ^{125}I-hGH per gel. In the more recent experiments, polyethylene glycol (PEG-6000) 25% in Veronal buffer (0.025 M, pH 8.6) was used instead of PAGE, in order to increase the number of replicates. The bound antigen was precipitated together with 50 μl of normal human serum by centrifuging for 20 minutes at approximately 3000 g. The equivalence between the two separation techniques was verified (unpublished data) by comparing their antibody titration and radioimmunoassay curves.

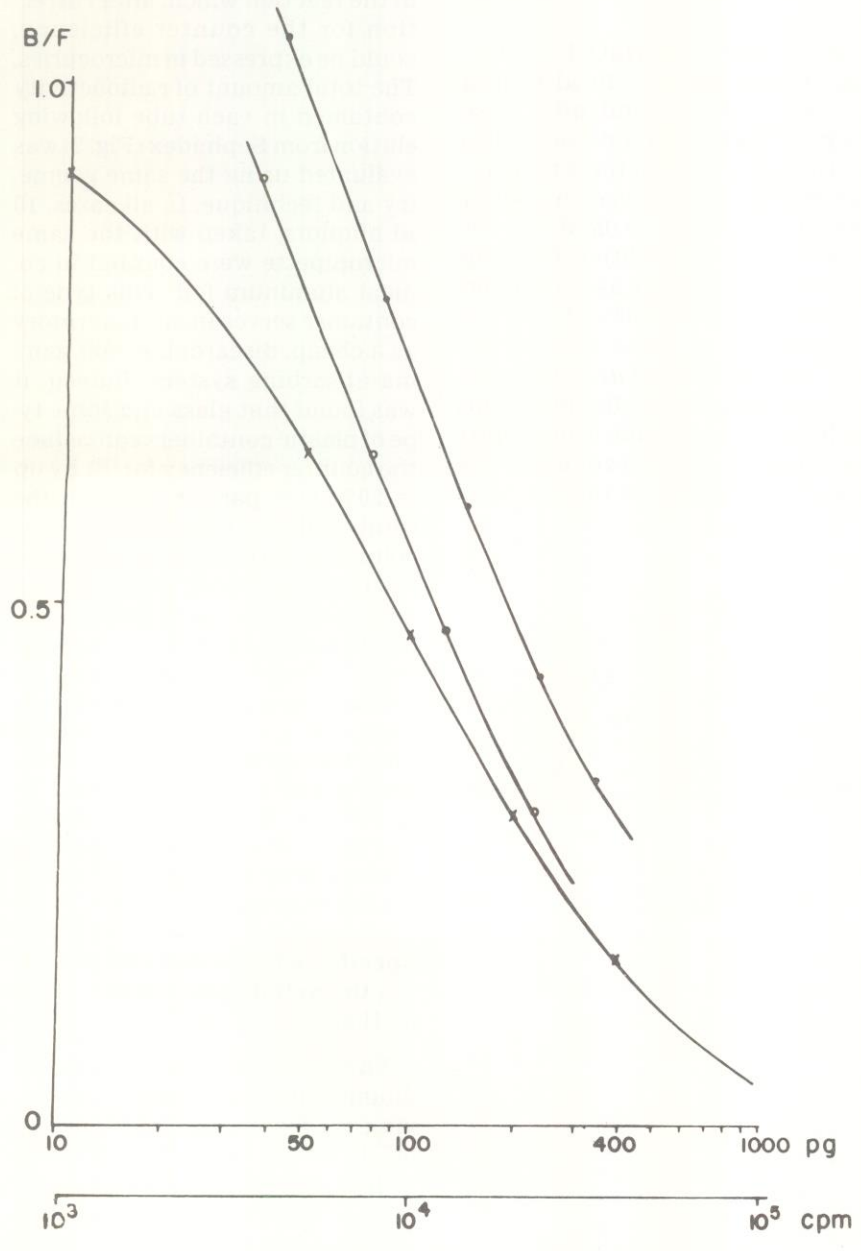

Fig. 3 — Self-displacement curves. — x — conventional standard curve relating B/F to picograms of added unlabelled hGH. • — "tracer curve" showing the decrease in B/F upon sequential addition of labelled antigen, measured in cpm_s. — o — curve obtained by adding the picograms of labelled hormone found to be present in each incubation to the corresponding point of the conventional standard curve.

RESULTS

As can be seen in Table 1, the coincidence method showed a stable counter efficiency of 80-82% over seven months and close agreement with the manufacturer's stated activity. A direct comparison between hGH and BSA labelling and chromatography (Fig. 2) and previous gel filtration studies of true and false labellings[4] led us to consider that, in addition to PII, only PIa and PIc correspond to hGH molecules, while PIb is radioactivity carried by BSA. The former (PIa) appears to be a type of aggregate of ^{125}I-hGH, while the latter (PIc) is probably labelled "big hGH". PII, PIa and PIc should therefore represent all the radioactivity bound to 5 μg of hormone used in the labelling; the specific activities shown in Table 2 were calculated based on this assumption. In Table 3, the specific activities measured by the two methods are compared for ten experiments carried out on preparations labelled at different specific activities, covering the range 10-200 μCi/μg. A simple linear regression analysis showed a highly significant correlation ($P < 0.001$) between the two methods.

In the self-displacement determination (Fig. 3) we observed that the mass of labelled hormone present in each incubation is not negligible with respect to the mass of unlabelled hGH and should be added to the latter to give the total mass of hormone on the abscissa; in this way parallelism between the two curves could be obtained.

Having found in previous work[3] that high specific activity tracers (100 μCi/μg) present a certain instability, an experiment on polyacrylamide gel electrophoresis (PAGE) was carried out to test the storage time effects on high and low specific activity tracers after an ^{125}I half-life (60 days). As can be seen in Fig. 4 a remarkable aggregation and de-iodination occurred in high specific activity ^{125}I-hGH at both -20°C and -196°C storage temperature. This practically did not occur with low specific activity ^{125}I-hGH stored at -20°C and

TABLE 3
Comparison of specific activities of iodinated hGH, calculated by the two methods

Exp. N.	^{125}I used in the labelling (μCi μg)	Self-displacement (sd) (μCi μg)	Analysis of reaction mixture (arm) (μCi μg)	arm sd^{-1}
1	1236	201.5	174.8	0.867
2	1155	195.0	163.7	0.839
3	994	98.3	100.0	1.117
4	858	94.5	88.5	0.936
5	755	106.6	93.9	0.881
6	694	119.1	94.5	0.793
7	522	41.9	35.1	0.838
8	397	48.2	40.1	0.832
9	360	31.1	33.3	1.071
10	183	14.0	14.5	1.036
			$\bar{x}$ =	0.911
			SD =	± 0.098
			CV =	10.8%

[1] **Linear regression analysis:**
arm = 0.837 sd + 4.240
($r = 0.993$ $\bar{p} < 0.001$)

TABLE 4
Study on the stability of low specific activity ^{125}I-hGH

Tracer	Specific Activity (μCi/μg)	Age of the tracer on assay date (days)	Spec. Bind. (%)	Non-spec. Binding (%)	ED_{50} of stnd. curve (ng/ml)	Intern. Quality Control (ng/amp)
A	25.4	21	27.2	9.2	9.0	26.9
		23	26.6	11.0	9.2	34.5
		24	31.7	11.5	9.0	43.2
		30	29.7	11.0	8.8	28.9
B	35.9	3	37.6	9.0	7.2	30.1
		10	34.9	7.3	6.9	31.2
		15	39.3	8.9	9.6	33.0
		16	37.7	11.8	8.0	40.0
		17	32.3	11.2	7.5	45.8
		21	38.0	11.6	6.5	38.7
		25	33.6	11.4	5.2	34.8
		66	34.2	9.0	7.6	26.6
C	22.6	35	36.8	9.9	7.2	30.8
		44	32.0	9.6	9.4	34.9
		46	29.3	9.6	12.3	34.2
		51	25.0	8.4	9.8	27.8
			$\bar{x}$ = 32.9	10.0	8.1	33.5
			SD = ± 4.4	± 1.3	± 1.3	± 5.7
			CV = 13.4%	13.0%	16.0%	16.8%

run under the same electrophoretical conditions.

For the reason mentioned above a sequence of labellings was carried out at low specific activities to test the reproducibility of our labelling and assay conditions. In these labellings, the same ampoulized preparation was used throghout, while a freshly opened ampoule was employed for each standard curve, fixing tracer and antibody concentrations as previously described[5]. Table 4 presents the results obtained preparing a tracer each month and following its immunological properties for up to two months via the analysis of some typical radioimmunoassay parameters: specific and non-specific binding, dose producing 50% fall in the maximum response (ED_{50}), dose related to an internal quality control sample.

DISCUSSION

Establishing standard labelling conditions has clearly produced satisfactory reproducibility in the labelling of the present protein hormone, allowing better control and prediction of specific activity of the product. This type of quality control is extremely important for improving inter-assay and inter-laboratory precision, still a serious problem in radioligand assays.

The percentages of ^{125}I actually found in the undamaged monomeric form of hGH, ranging from 51 to 58% in ten experiments with a coefficient of variation of 5.3%, exhibit an agreement and reproducibility seldom reported in protein radioiodination. This may be due to the fact that we had access to freshly prepared and dissolved hGH. According to our experience, in fact, most of the problems encountered in protein labelling are related to the type, age or storage conditions of the unlabelled hormonal preparation. The present results also indicate that, unlike the fundamental work of Greenwood et al.[19], under our operating and counting conditions there are no great losses in radioactivity, and consequently (we assume) in protein, at any point during the entire labelling and purification process and that the "damaged" components are not as highly adsorbed to Sephadex as reported.

The agreement between our approach to specific activity determination (arm) and the immunological method (sd) is, in our opinion, quite acceptable for practical purposes, especially considering the extremely low concentrations and the different mechanisms on which the two methods are based. It must be pointed out that the determinations were carried out shortly after the labelling and purification, indicating that our radioiodination conditions do not appear to alter the immunological properties of the tracer as was found by Verhoff et al.[35] in one of his experiments.

A possible explanation of the slightly higher values obtained with the self-displacement technique can be found in the well known mechanism called "decay catastrophe" which can be produced by ^{125}I intra-molecular decay effects[7]. An immunological inactivation of hGH molecules subjected to this catastrophe, would consequently produce higher specific activities, when determined by this technique, especially for high iodination degrees. This seems indeed the tendency indicated in Table 3 and confirmed by the experiment presented in Fig. 4. In fact, PAGE analysis of the storage effects shows, as previously described[3], that these are indeed of nu-

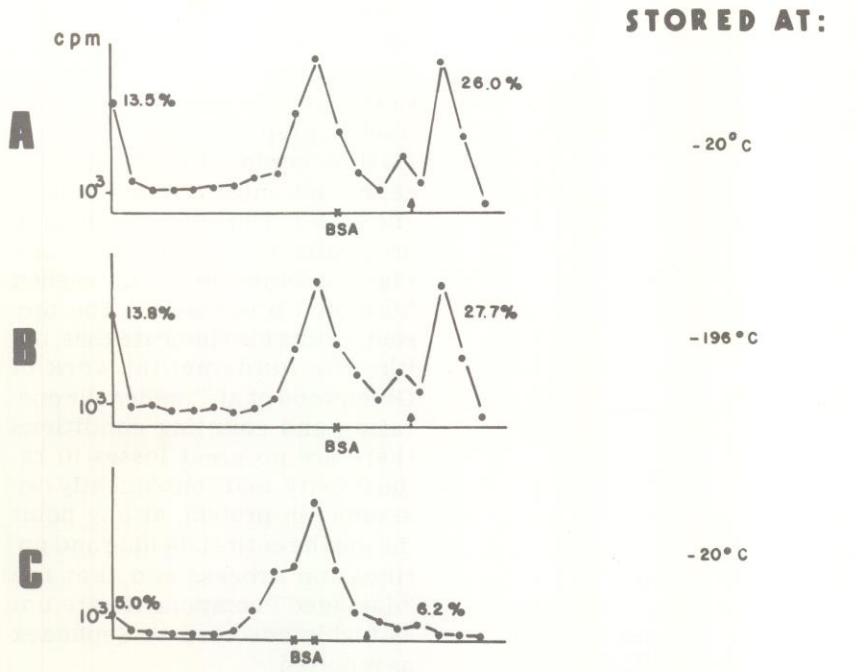

Fig. 4 — Evaluation of storage time (^{125}I $T_{1/2}$ = 60 days) effects on high and low specific activity tracers, run on PAGE.
A) High specific activity ^{125}I-hGH (107 $\mu Ci/\mu g$) stored at -20°C.
B) The same as in A), but stored in liquid nitrogen at -196°C.
C) Low specific activity ^{125}I-hGH (14 $\mu Ci/\mu g$) stored at -20°C.
The three main peaks from left to right are: ^{125}I-hGH aggregate, monomeric ^{125}I-hGH and free ^{125}I.
The arrows indicate the tracking dye position.

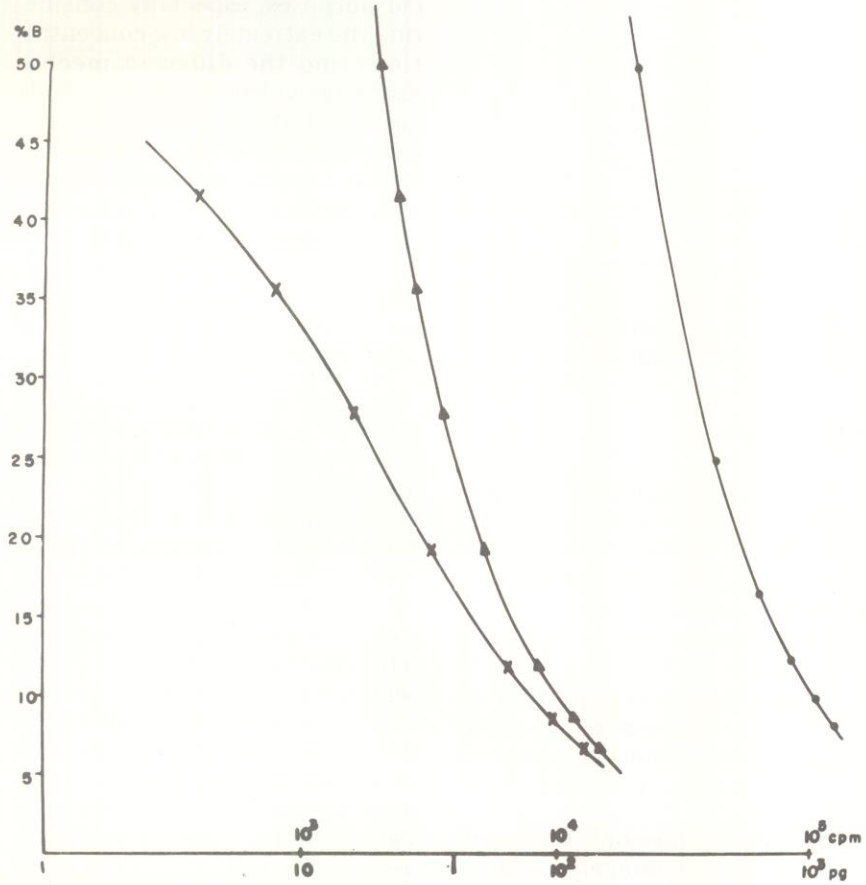

Fig. 5 — Analysis of parallelism between the self-displacement curves theoretically calculated by Roulston.
— x — Conventional standard curve. — ▲ — Curve obtained adding 20 pg of tracer (as in hypothesis) to each point of the first curve. —·●— Tracer curve (cpm versus % bound).

clear origin, since their kinetic is completely unaffected by extremely low temperatures (-196°C). This is obviously much more evident for high specific activity tracers, considered their increased number of polyiodinated molecules[7]. Our results therefore differ somewhat from those for hCG, hLH and hTSH presented by Englebienne et al.[17] and from those for ^{125}I-Thyroxine presented by Bhupal et al.[10], who attributed the lack of agreement between the two methods to the presence of unreactive impurities or to non-specific Thyroxine adsorption. In our case these impurities were probably either not present or eliminated by the purification process, while tracer loss was extremely reduced as one can observe from the total recovery values presented in Table 2.

The preliminary study on inter-assay reproducibility using different tracers indicated that the low specific activity ^{125}I-hGH prepared and characterized with the present method is stable over the period of at least two months and can be prepared from the same ampoulized hGH preparation, providing radioimmunoassay curves whose inter-assay precision, calculated over a long period of time, is quite satisfactory for protein hormones.

Concerning the controversy in the literature regarding the validity of the self-displacement technique, we have confirmed, through comparative data analysis, the equivalence of two reported[18, 29] methods of calculation as stated by Roulston[33]. However, we observed a lack of parallelism between the two self-displacement curves which is much more evident in our case (Fig. 3) than in the data of Morris[29]. Since ii has not been mentioned by any of the authors referred to above, we emphasize its experimental and theorical explanation. In fact each point of the first curve (addition of unlabelled hGH) does not take into consideration the fixed mass of hGH due to the tracer, while the second curve (addition of ^{125}I-hGH) does. Upon adding this mass to each

point of the first curve, parallelism is obtained, showing the identical immunological behaviour of the labelled and unlabelled preparation. Applying these considerations to Roulston's theoretical curve, an identical result was obtained (Fig. 5).

Finally the coincidence method has proved to be very useful; in addition to being simple, extremely flexible and precise (0.87% coefficient of variation in counter efficiency determination over a seven month period), no additional equipment is required (even by a small laboratory for clinical assays) and the periodic purchase of calibration standards is avoided. Routine use of this method has confirmed not only the stability of our counter efficiency, but also the excellent conservation of $Na^{125}I$ lots[6], no significan losses due to wall adsorption or evaporation being noted even after shipping delay and weeks of storage and use.

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RESUMO

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BARTOLINI, P. e col. — Aprimoramento da reprodutibilidade e dos controles de qualidade na marcação do hormônio de crescimento humano com ^{125}I. **Rev. Hosp. Clín. Fac. Med. S. Paulo 43**(2): 93-100, 1988.

A reação de marcação do hormônio de crescimento humano (hCH) com ^{125}I e sua purificação cromatográfica foram estudadas focalizando-se especialmente a reprodutibilidade dos rendimentos, as recuperações quantitativas e as atividades específicas obtidas. Através da rigorosa padronização de uma técnica de monitoração foi confirmado que não há perdas significativas de radioatividade ou proteína durante o processo de marcação ou purificação. Controlando rigorosamente as condições de reação foi obtida uma reprodutibilidade satisfatória na marcação de vários extrados de hCH usando diferentes embarques de ^{125}I após curtos ou longos períodos de estocagem deste radioisótopo. Finalmente a atividade específica (ou massa absoluta) da proteína radioiodada foi determinada mediante a análise da mistura de reação, sendo comparada àquela obtida pelo método radioimunológico (self-displacement) mais universalmente usado.

Para poder expressar as atividades específicas diretamente em microcuries por micrograma, uma técnica simples de contagem em coincidência, que permite a determinação da atividade absoluta de fontes de ^{125}I, foi adaptada às nossas condições laboratoriais.

A purificação e os controles de qualidade descritos permitem a obtenção de produtos marcados de boa reprodutibilidade, o que é indispensável para alcançar uma maior precisão inter-ensaio e inter-laboratorial.

Unitermos: **Radioiodação. Hormônio de Crescimento Humano (hCH). ^{125}I. Radioimunoensaio. Atividade Específica.**

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