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The use of recombinant human growth hormone for radioiodination and standard preparation in radioimmunoassay

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Recombinant human growth hormone (rec-hGH) obtained by cloning hGH precursor gene, bacterial expression and periplasmic secretion of the authentic, mature form of the hormone was used, after purification and characterization, for the preparation of radoimmunoassay (RIA) reagents. ^{125}I-rec-hGH was prepared by the classical chloramine-T iodination technique, while an internal standard of the same rec-hGH was used and calibrated against pituitary hGH reference preparation (NIDDK-hGH-RP-1) with the use of a reference antiserum (NIDDK-anti-hGH-2). In both cases the behavior of the recombinant preparation was identical to that of the pituitary hormone. This confirms previous data on bacterial correct processing and folding of the protein, as far as its immunological behavior is concerned and indicates its suitability for the preparation of immunoassay reagents.

Key words: Human growth hormone; Recombinant; Radioimmunoassay; Radioiodination; Reference preparation

Introduction

Recombinant techniques have already presented innumerous applications in the production of proteins of medical interest, for therapy and diagnosis. In the case of human pituitary hormones many different approaches have been used for the cloning and expression of these proteins in bacteria and other prokaryotic or eukaryotic host cells. This is especially the case for human growth hormone (Goeddel et al., 1989; Gray et al., 1984; Ikehara et al., 1984; Kato et al., 1987) whose importance in the treatment of pituitary dwarfism is widely recognized. Other anterior pituitary hormones (human thyroid-stimulating hormone, human luteinizing hormone, follicle-stimulating hormone; human prolactin) have also been cloned and expressed by various methods (Simon et al., 1988; Wondisdorf et al., 1988; Keene et al., 1989; Paris et al., 1990), but only in a few cases has the purified and characterized final product been obtained (Simon et al., 1988; Paris et al., 1990; Kashiwai et al., 1991).

Most of the interest in this work has been related to gene expression mechanisms and structure-function relationships or, from a clinical point of view, to therapeutic approaches and uti-

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lization for in vitro diagnostic techniques which have very seldom been studied (Kashiwai et al., 1991; Thotakura et al., 1991).For this reason in this paper we have studied the utilization of rec-hGH for the preparation of radioimmunoassay (RIA) reagents, considering also that this recombinant protein had already been produced, purified and characterized in our laboratory for therapeutic applications (Bartolini et al., 1990). We also consider this a first step in the utilization of other recombinant pituitary hormones, thereby avoiding, not only the ethical and legal problems frequently related to human pituitary collection, but also the potential contamination hazards related to their handling and processing. It is also worth noting that in many cases pituitary hormone extraction and purification is a long and tedious process, mainly because these proteins are often structurally and physicochemically very similar. Reagent reproducibility and continuity, together with the avoidance of such interferences, are clear advantages related to the utilization of DNA recombinant products for this purpose.

Materials and methods

Recombinant human growth hormone was prepared and purified in our laboratory (Bartolini et al., 1990). Part of the molecular biological work, starting from poly(A)$^+$ RNA extracted from frozen pituitary tissue and used for the construction of a cDNA pituitary library, was carried out in the Istituto di Genetica, Biochimica ed Evoluzionistica-CNR (Pavia, Italy). Some of the physico-chemical purity, potency and identity tests on the final product were carried out at the National Institute for Biological Standards and Control (South Mimms, Hertfordshire, UK) in collaboration with Dr. D. Schulster. Its biological activity,determined in a body weight gain assay in hypophysectomized rats, was not significantly different from that presented by the 1st International Standard of hGH, WHO 80/505, whose declared potency is 2.59 IU/mg. Pituitary human growth hormone reference preparation (NIDDK-hGH-RP-1) and antiserum (NIDDK-anti-hGH-2) were kindly provided by the National Hormone and Pituitary Program (Baltimore, MD, USA). Highly purified pituitary human growth hormone (pit-hGH) for radioiodination was purchased from Dr. P. Torjesen (Aker Hospital, Oslo, Norway), while goat anti-rabbit second antibody was obtained from ICN (Carson, CA, USA). Sephadex G-100 was a product of Pharmacia-LKB, while $Na^{125}I$, free of carriers and reductants, was purchased from Medgenix Diagnostics (Wevegelm, Benelux) with a specific activity of 200–300 mCi/ml (7400–11,100 MBq/ml).

Radioiodination

The ^{125}I-labelling of pit-hGH and rec-hGH was carried out using a modification (Biscayart et al., 1989) of the original Chloramine-T technique (Greenwood et al., 1963) employing 0.5–0.7 mCi (18.5–25.9 MBq) of radioisotope, 5 μg of hormone in 30 μl 0.3 M potassium phosphate buffer pH 7.4 and 0.8 μg of chloramine-T in 12 μl 0.05 M phosphate buffer 7.4. The reaction proceeded at room temperature for 5 min, adding then 1 μg sodium metabisulfite in 5 μl of the same 0.05 M phosphate buffer. Tracer purification was performed by gel filtration on Sephadex G-100, used in a preparative and analytical way, following the conditions described in previous work (Bartolini et al., 1986) which allows a precise calculation of the distribution coefficient (K_D) of all radioactive peaks. The use of this parameter (which is related to the protein Stokes radius) together with the purification process, may provide a purity and identity test (Ribela et al., 1988). A set of titration curves using 0.1 ng/ml and 1.0 ng/ml of ^{125}I-hGH and 0.1 ng/ml ^{125}I-hGH + 0.9 ng/ml of hGH, as described by Hunter (1973), was run to ascertain the identical immunological activity of radioiodinated and unlabelled hGH.

Radioimmunoassay

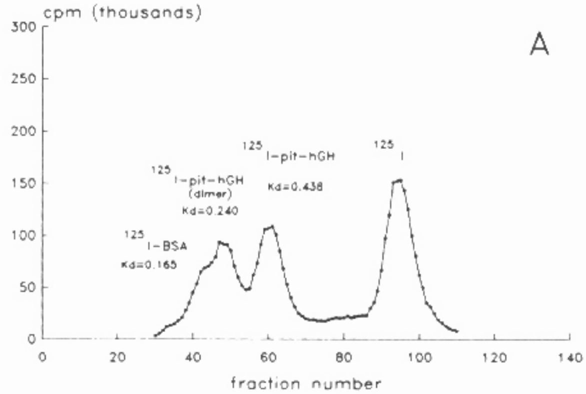
RIAs were carried out with simultaneous addition of all reagents: tracer (25,000 cpm), reference preparation and first antibody (1/300,000 final dilution). Incubation was carried out at 4°C for 24 h in phosphate buffer 0.01 M pH 8.6 with 0.1% bovine serum albumin (BSA) using a liquid phase second antibody technique, with 1.5 h incubation at room temperature, for the separation of the antibody bound hormone. In each assay low, medium and high dose quality control samples

(QCS) prepared with pit-hGH were always run together with the standard curve. Unknown serum samples were determined when studying the correlation between the use of the two tracers or reference preparations. Sensitivity was calculated according to Rodbard's (1978) definition.

Polyacrylamide gel electrophoresis (PAGE), for the quality control of the two tracers through their mobility ratio (R_m) determination, was performed as previously described (Bartolini et al., 1977).

Results and discussion

In Fig. 1 is shown a comparison between the purification of radioiodinated pit-hGH and rec-hGH. In Fig. 1*A* a specific activity of 46.2 μCi/μg of protein was obtained while in Fig. 1*B* it was 43.5 μCi/μg (see also Table I). The routine radioiodination conditions used were those required to provide low specific activity tracers. In this way one can obtain tracers that combine good quality binding properties and long term stability. The reproducibility of the distribution coefficient (K_D) values for both labelled preparations may be seen in Figs. 1*A* and 1*B* (0.438 for ^{125}I-pit-hGH and 0.451 for ^{125}I-rec-hGH). Recent cumulative statistics, for monomeric ^{125}I-pit-hGH indicate, on the same chromatographic column, a $K_D = 0.445 \pm 0.029$ SD (for $n = 25$). The two peaks observed (Fig. 1) with K_D of 0.165 and 0.189 correspond to ^{125}I non-specifically bound to carrier BSA, an undesired component described in previous work (Bartolini et al., 1981). Statistical calculations for ^{125}I-BSA show a $K_D = 0.166 \pm 0.026$ ($n = 22$). In the case of the pituitary preparation a second large peak, whose elution volume is closer to monomeric hGH, can be seen. Its $K_D = 0.240$ identifies it as a dimeric form. Previous work carried out with gelatin instead of BSA as carrier, allowed the precise identification of a dimeric hGH, appearing only in some preparations, and whose K_D was 0.261 ± 0.027 ($n = 8$)



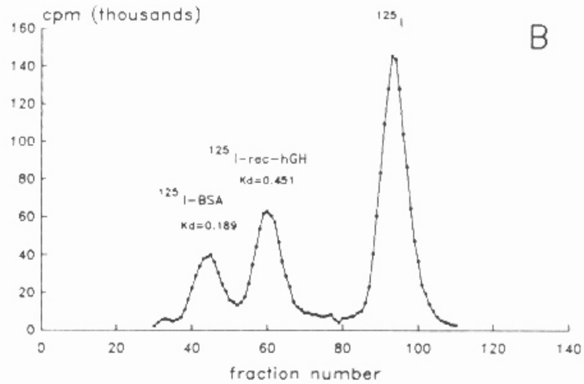


Fig. 1. Purification and analysis of radioiodinated products on a 2.5×50 cm Sephadex G-100 column; flow rate 12 ml/h. *A*: ^{125}I-pit-hGH. *B*: ^{125}I-rec-hGH.

TABLE I

A COMPARISON BETWEEN SOME TYPICAL PARAMETERS RELATED TO pit-hGH AND rec-hGH RADIOIODINATION

Hormonal preparation	Reacting protein (μg)	Reacting ^{125}I (μCi)	Labelling yield (%)	Specific activity (μCi/μg)	K_d of radioiodinated peaks 1st	2nd	3rd	R_m	Specific binding to antibody 1/300,000 (%)
pit-hGH	5.0	573	40.3	46.2	0.165	0.240	0.438	0.79	44.6
rec-hGH	3.5	624	24.5	43.5	0.189	–	0.451	0.76	45.4

TABLE II

UTILIZATION OF ^{125}I-pit-hGH AND ^{125}I-rec-hGH IN THE ASSAY OF UNKNOWN SERUM SAMPLES

Assay no.	Number of assayed samples	Correlation curve	
		Slope	Corr. coefficient
1	28	1.08	0.970
2	18	1.00	0.998
3	13	1.07	0.097
4	14	0.98	0.967

(Ribela et al., 1984). In the case of the pituitary hGH preparation the dimer might have been produced as a consequence of the long storage period of the dissolved preparation. In fact the rec-hGH, which here was freshly prepared and immediately labelled, presented a considerable amount of dimer after about one year storage, even at −80°C. A very small amount of aggregate, running right after the void volume, was present in both preparations.

In Table I we report some typical parameters that are routinely determined for the quality control of our tracers. We also determined the mobility ratio (R_m), running the purified tracers on 7% native PAGE. In Table II we can see the results following use of these two tracers for the determination of $n = 73$ unknown serum samples in a RIA system using pit-hGH as reference preparation. A good correlation and practically no bias (slope proximal to unity) between the two systems can be observed. The stability of ^{125}I-rec-hGH was confirmed over a 2 month storage at −20°C in comparison with ^{125}I-pit-hGH (Table III).

When pit-hGH and rec-hGH were used as reference preparations in a RIA system using ^{125}I-pit-hGH as tracer a good superimposition between the two standard curves could be observed (Fig. 2*A*). Pit-hGH (NIDDK-hGH-RP-1) reference preparation was dissolved as recommended, using doses according to the declared content. In the case of rec-hGH, doses were set up according to the determined protein content (Lowry et al., 1951). The relative potency of the two preparations was determined in four independent assays using curve parameters (ED_{50}, ED_{20} and ED_{80}) after having run a test for parallelism (Rodbard et al., 1978). This calibration provided a relative potency of rec-hGH against

TABLE III

COMPARISON BETWEEN THE STABILITY OF ^{125}I-rec-hGH AND THAT OF ^{125}I-pit-hGH DURING STORAGE AT −20°C OVER A 2-MONTH PERIOD

Tracer life (days)	Specific binding to antibody 1/300,000 (%)	
	^{125}I-pit-hGH	^{125}I-rec-hGH
11	44.6	45.4
30	34.1	34.8
46	34.6	35.4
62	31.8	32.6

Correlation curve comparing ^{125}I-pit-hGH and ^{125}I-rec-hGH specific bindings: $r = 1.000$; slope = 1.002; intcp = 0.694; ($P < 0.001$).

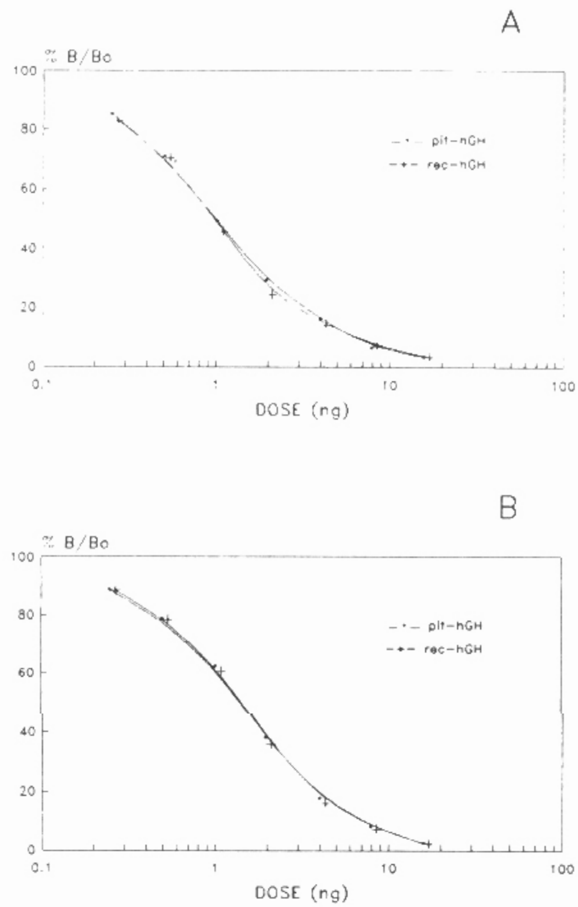


Fig. 2. Radioimmunoassay standard curves with the use of pit-hGH and rec-hGH as reference preparations. B/B_o = bound counts/maximum binding. The tracers were: *A*: ^{125}I-pit-hGH; *B*: ^{125}I-rec-hGH.

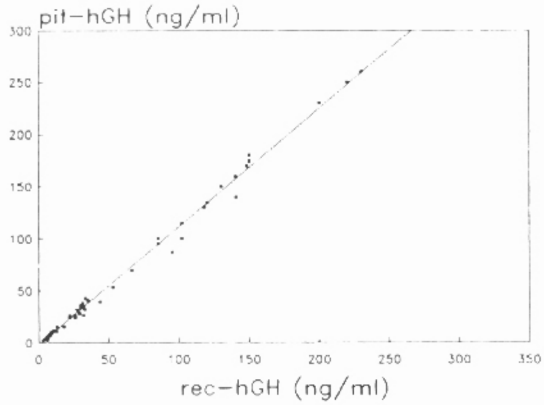


Fig. 3. Linear regression showing the comparison between pituitary and recombinat hGH reference preparations for the determination of $n = 53$ unknown serum samples and pituitary extracts, using ^{125}I-pit-hGH as tracer.

pit-hGH (NIDDK-RP-1) of 1.037 ± 0.063 (inter-assay CV = 6.1%), proving also the equivalent immunological activity of the two purified preparations. The same study was carried out also using ^{125}I-rec-hGH as a tracer (Fig. 2*B*), providing for rec-hGH reference preparation a relative potency of 1.024 ± 0.087 (CV = 8.5%, for $n = 4$ assays) The sensitivity of the assay system was calculated as 0.3 ng/ml (minimal detectable concentration).

In Fig. 3 the correlation curve obtained with the use of the two different reference preparations in a system using ^{125}I-pit-hGH as tracer in the determination of unknown serum samples and pituitary extracts ($n = 53$) is presented. The correlation coefficient was $r = 0.985$ while practically no bias is introduced (slope = 0.9998) when using rec-hGH reference preparation instead of NIDDK-RP-1. We deliberately expanded the dose-response curve up to more than 200 ng/ml in order to check its linearity for concentrations well above the usual serum determination range, particulary useful for pituitary or bacterial extract determination.

In conclusion, we described the use of purified authentic recombinant hGH for the purpose of immunoassay reagent preparation: standard and radioiodinated hormone It is interesting to observe that the immunological activity of purified rec-hGH was identical to that of pit-hGH (NIDDK-RP-1) while its radioiodination produced a tracer practically identical to that obtained with a well known purified commercial preparation. This is quite relevant, especially considering that one of the main problems in immunoassay reagent preparation and standardization, together with the type of antibody being used, is the frequent existence of 'isoforms' (Ekins, 1992). These 'isoforms', or 'isohormones', in the case of hGH and other pituitary hormones can be present in either the pituitary extract or recombinant product, due to different post-transcriptional or post-translational mechanisms, or originated by extraction artefacts; they may have very different immunological activities (Lewis, 1978). The availability of a well characterized expression vector and bacterial strain, together with a standardized purification process and adequate physico-chemical quality control (especially based on size exclusion and reversed-phase HPLC), can provide a practically unlimited amount of this and other hormones, leading to a better controlled reagent continuity without further need of organ collection.

We preferred not to include, in the present study, the variable 'antibody', and therefore we did not prepare, for comparison, an antiserum against rec-hGH. In our opinion, different antibodies are the most common causes of bias, especially between-laboratory and between-methods, even when the same source of antigen is used. For this reason, in our study, a well known (standard type) antibody preparation from NIDDK was used. However, we believe that antibodies, preferably monoclonal, produced against purified preparations (22 K hGH, for example) of recombinant or pituitary hormone, should be equally useful as immunoassay reagents. For reagent continuity and better interassay and interlaboratory reproducibility, in our opinion, a monoclonal antibody (or the corresponding hybridoma) directed against a well characterized form, could be considered for distribution as an internal and external reference preparation.

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