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COMPARISON BY GEL FILTRATION CHROMATOGRAPHY AND REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF THE IMMUNOREACTIVE GROWTH HORMONE COMPOSITION OF A HUMAN PITUITARY EXTRACT

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SUMMARY

The immunoreactive growth hormone composition of a pituitary extract has been compared by conventional gel filtration chromatography (pH 8), and reversed-phase high-performance liquid chromatography (pH 2) on a wide-pore (300 A) short-chain column. By gel filtration chromatography, four peaks of immunoreactivity were obtained, labelled "monomer", "dimer", "aggregate" and "void". However, by high-performance liquid chromatography all of these fractions were themselves shown to be multicomponent mixtures. The "monomer" peak contained at least two forms (M_1 and M_2). The "dimer" fraction contained three peaks, two of which co-eluted with M_1 and M_2, and a third component, D. Similarly, the aggregate fraction contained M_1, M_2, D and a fourth component, A. The "void", in contrast, contained mostly M_1 and M_2 with very little D. One interpretation of these results is that M_1 (the 22K molecular weight monomeric form) and M_2 (a chemically modified form of M_1) are present in all molecular weight fractions in loosely bound aggregates which break up under acidic conditions. D and A are probably oligomeric forms of growth hormone (possibly a dimer and higher molecular weight species, respectively).

INTRODUCTION

In recent years high-performance liquid chromatography (HPLC) has been used extensively for the purification and analysis of protein and peptide hormones (see ref. 1–4 for reviews). However, application of this technique to the separation of growth hormone (GH) variants* has received little attention, even though the known forms differ in both mass and charge[5].

The principal GH component in both human pituitaries and plasma is the so-called "22K" monomer (hGH_{22k}). Other variants isolated from pituitary extracts

* The term "variant" is used to include variants, post-translational modifications and fragments as defined by Lewis *et al.*[5].

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and/or plasma include hGH_{20k} (hGH_{22k} minus amino acids 32–46), cleaved monomeric forms (either disulphide bridge or sequence cleavages), deamidated and acetylated variants, alkaline forms, and dimeric and possibly larger oligomers (both covalently bonded and loosely aggregated)[5,6]. Some of these components may be experimental artifacts.

The main chromatographic techniques used in purification of hGH have been conventional gel filtration (G100 and G200)[7], affinity[6] and anion-exchange chromatography[8]. Reversed-phase HPLC has been used only for mapping of tryptic digests of hGH_{22k} and hGH_{20k}[9], to purify tumour hGH_{22k}[10] and in a partially successful attempt to separate hGH_{22k} and hGH_{20k}[11].

This paper represents the first part of a study designed to apply the high resolution, and wide range of separation modes, available from the current generation of HPLC column packing materials to the analysis of hGH variants. In this case, the immunoreactive composition of hGH, already partially purified from a pituitary extract, was compared by conventional gel chromatography and HPLC using a reversed-phase short-chain (RPSC) column.

EXPERIMENTAL

Partially purified pituitary hGH

The hGH used had been purified in a large-scale preparation as reported previously[8]. Briefly, this technique involved separation by gel filtration chromatography, with further purification by anion exchange chromatography. The purified material had been freeze dried and stored at −20°C for several years prior to this study. The precise composition of the material used in this study was unknown.

Gel filtration chromatography

Gel filtration chromatography was performed on a column (100 × 1.5 cm I.D.) of Sephadex G100 superfine (Pharmacia) eluted with 0.05 *M* NH_4HCO_3–0.1% (w/v) human serum albumin (HSA)–0.1% (w/v) sodium azide buffer (pH 8.0) at 3 ml h^{-1}. Fractions were collected for 40 min each (*ca.* 2 ml). The void volume was marked with bovine thyroglobulin (669,000 MW) and the salt peak by potassium iodide (visualised by addition of acidified hydrogen peroxide). Typically, hGH (200 ng) was loaded in 0.5–1.0 ml of buffer.

HPLC

HPLC was carried out on a Varian 5560 fitted with an Ultrapore RPSC column (75 × 4.6 mm I.D.) (Altex). This is a wide-pore (300 A) 5 μm silica-based column with a propyl bonded phase. Elution was performed using water–0.1% (v/v) trifluoroacetic acid (TFA) (Solvent A; pH 2) and 1-propanol–0.1% (v/v) TFA (Solvent B), with a gradient from 26 to 35%B over 27 min at 1 ml min^{-1}. Typically 200 ng of hGH in 200 μl of solvent were loaded via a Rheodyne 7125 loop injector. Detection was at 280 nm and the signal was recorded on a dual pen chart recorder (JJ Instruments). Fractions were collected (1.06 ml each) with a Frac 300 (Pharmacia) into plastic tubes, containing 1 ml of 0.1% v/v Triton X-100 aqueous solution to prevent hormone adsorption to the tube. In one case, two larger scale injections of hGH (75 and 180 μg) were made, using a separate RPSC column on an Altex HPLC system,

but with the same solvents. In this case, a 421 controller was used to programme two 100A pumps linked to a 210 injector. Detection was via a Hitachi 155-00 detector at 280 nm, recorded on a Shimadzu C-R1A integrator. Gradients were 26–35%B in 18 min and 30–40%B in 30 min for the 180 and 75 μg injections, respectively. Fractions corresponding to the observed peaks were collected and immediately re-injected separately to ensure that each component recorded on the chromatogram was authentic and not an on-column breakdown product.

Reagents

Water was distilled and then purified further through a Milli-Q four cartridge system (Millipore). 1-Propanol and TFA were HPLC-grade (Rathburn Chemicals), whilst all other chemicals were AnalaR-grade (BDH) or better.

Radioimmunoassay

Fractions from both G100 chromatography and HPLC were quantified by radioimmunoassay (RIA). Initially, tubes were dried by vacuum desiccation over self-indicating silica gel–sodium hydroxide pellets and then reconstituted in assay buffer (0.05 *M* phosphate, pH 7.4, 2% (v/v) horse serum, 0.1% (w/v) sodium azide). Then 50 μl of each fraction were diluted to 400 μl with assay buffer, and 50 μl of a sheep antibody were added (initial dilution 1:92000). After a 24-h incubation, 50 μl of [^{125}I]hGH (*ca.* 12.5 nCi/0.25 ng) were added and left for a further 24 h. Precipitation of the antigen–antibody complex was effected with either a support-coated second antibody (Sac-Cel; Wellcome) or a polyethylene glycol (PEG 6000) assisted second antibody (donkey antisheep) and centrifugation. Standards were prepared from the WHO First International Reference Preparation (IRP) (66/217) by dilution in 0.05 *M* phosphate buffer–0.5% (w/v) HSA–0.1% (w/v) sodium azide (pH 7.4) and stored at −20°C.

RESULTS

Gel filtration chromatography

Partially purified hGH was analysed by G100 chromatography and the chromatogram of fraction number *vs.* concentration is shown in Fig. 1. The major component of immunoreactive hGH (ir-hGH) eluted in the same position as monomeric [^{125}I]hGH and the main peak of the WHO First IRP for RIA, and was thus deemed to be monomeric growth hormone (this would include the 20K form, if present). There are also three other discrete peaks present; two have K_{av} values about twice and three times that of monomeric hGH, whilst the third eluted in the void ($> 10^5$K). The four components were therefore named for convenience as "void" (8.2%), "aggregate" (2.5%), "dimer" (12.2%) and "monomer" (77.1%) in order of elution. Figures in brackets refer to the precentage of total ir-hGH recovered that was present in each peak.

HPLC

Fig. 2 shows the UV_{280} trace of a 75-μg injection of the original, partially purified material. Again four discrete peaks (1–4) are visible, with one major component (peak 1; 69.4%) and three lesser forms (peaks 2–4; 21.3%, 6.5% and 2.8%,

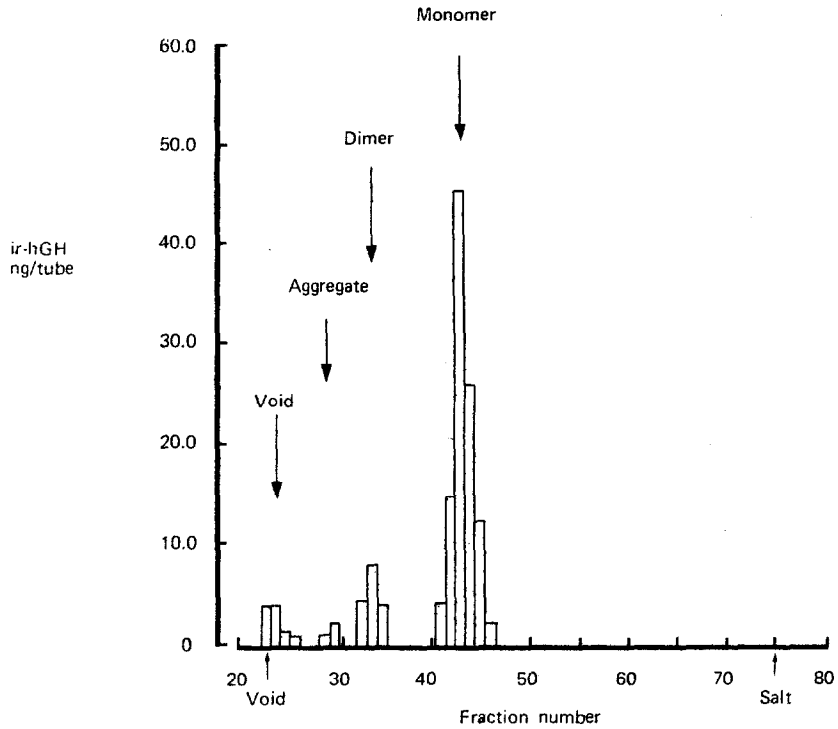


Fig. 1. Immunoreactivity profile of partially purified human pituitary growth hormone after gel filtration chromatography. Elution conditions: 100 × 1.5 cm I.D. G100 Superfine eluted with 0.05 *M* NH_4HCO_3–0.1% HSA–0.1% sodium azide. Fractions (2 ml) were collected and assayed by RIA.

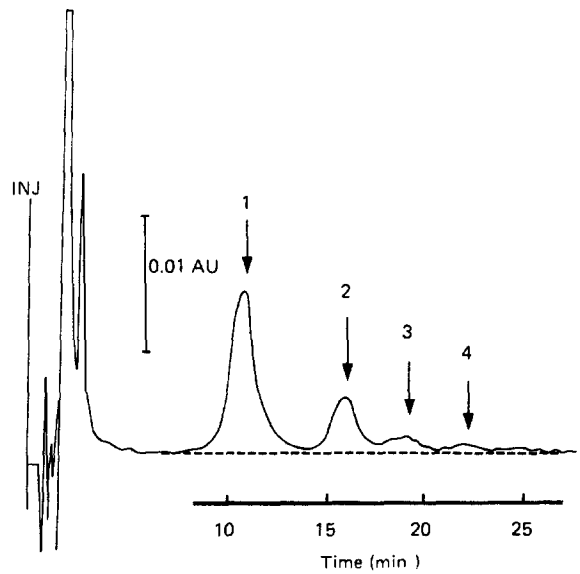


Fig. 2. Chromatogram of partially purified growth hormone (75 μg) after reversed-phase HPLC on an Ultrapore RPSC column. Gradient conditions: 30–40% B in 30 min. Solvent A, water–0.1% (v/v) TFA; Solvent B, 1-propanol–0.1% (v/v) TFA. Detection at 280 nm.

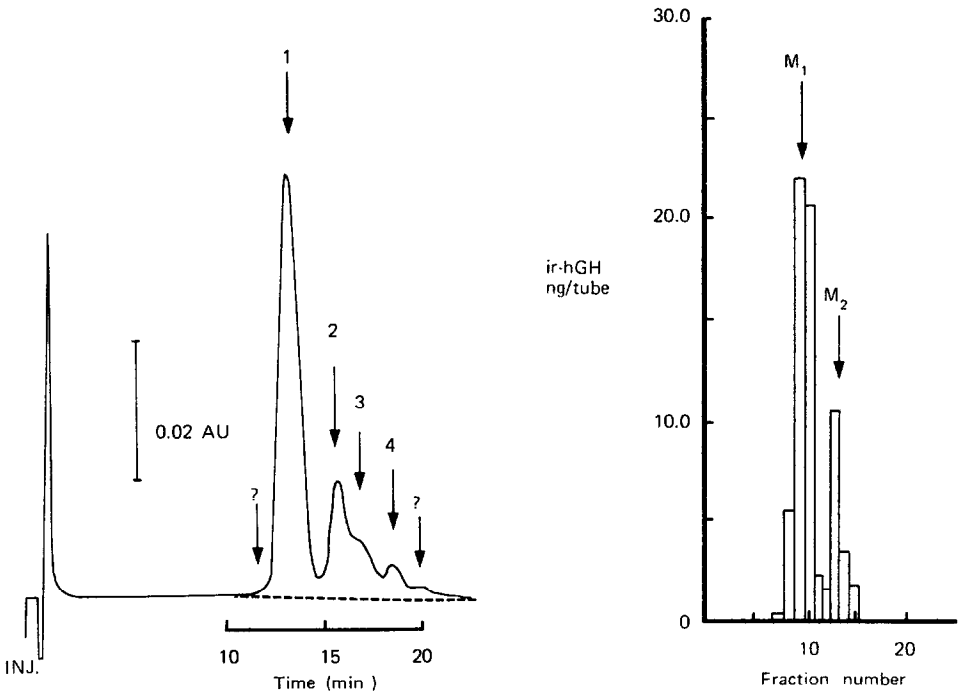
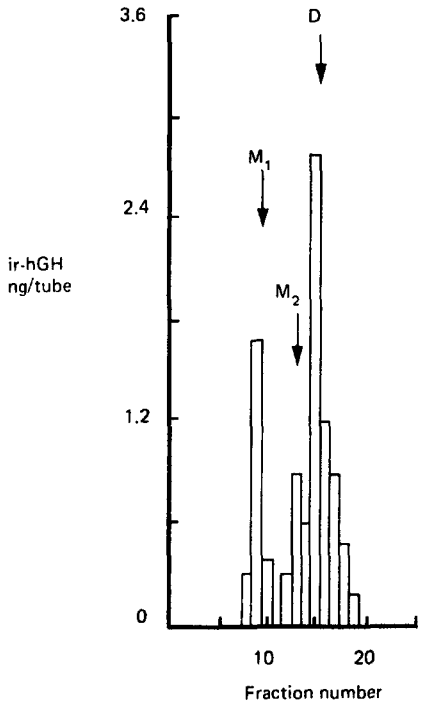


Fig. 3. Chromatogram of partially purified growth hormone (180 μg) after reversed-phase HPLC. Conditions as in Fig. 2, except 26–35% B in 18 min.

Fig. 4. Immunoreactivity profile of "monomer" fraction after reversed-phase HPLC. Conditions as in Fig. 2, except 26–35% B in 27 min; 1.06-ml fractions collected and analysed by RIA.

respectively). That these four components are genuine, and not column artifacts, was demonstrated by immediate re-injection of each peak. In each case, one peak only, at the correct retention time, was obtained. In addition, each component contained ir-hGH when assayed. A second large-scale injection (180 μg), run on a steeper elution gradient, indicated the possible presence of two further minor components, one eluting prior to the main peak and the other after the last peak (Fig. 3).

Tubes containing ir-hGH corresponding to each peak on G100 chromatography were then combined, and the "monomer", "dimer", "aggregate" and "void" fractions were analysed separately by HPLC. Fig. 4 shows the ir-hGH chromatogram of the monomer fraction. Two components are present, which co-elute with 1 and 2 in Fig. 2. These are therefore labelled M_1 and M_2 in Fig. 4 to indicate their origin from the monomer peak from G100 chromatography. The dimer HPLC trace in Fig. 5 is more complex. The main peak co-elutes with 3 in Fig. 2 and is re-labelled D to indicate its mainly dimer origin. However, significant amounts of M_1 and M_2 are also present, and so is a possible fourth component in the trailing edge of D. The HPLC trace for the aggregate fraction is equally complicated (Fig. 6). The two main components are M_1 and a component which co-elutes with 4 in Fig. 2. This was re-labelled A to indicate its mainly aggregate origin. Note that small amounts of M_2 and D are also present and even the possibility of a fifth, later-eluting component. In complete contrast, the void fraction is very simple (Fig. 7). The component M_1 comprises the majority of the ir-hGH, with lesser amounts of M_2 and D.



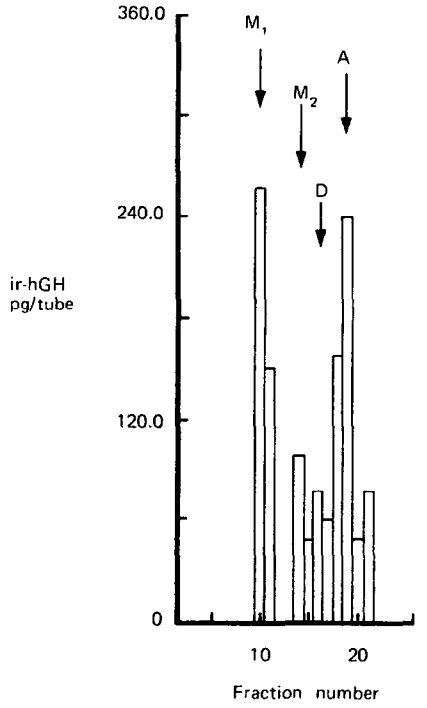


Fig. 5. Immunoreactivity profile of "dimer" fraction after reversed-phase HPLC. Conditions as for Fig. 4.

Fig. 6. Immunoreactivity profile of "aggregate" fraction after reversed-phase HPLC. Conditions as for Fig. 4.

These results are summarised in Table I. The totals given refer to the percentage relative proportions of each component present by G100 chromatography (top row) and HPLC (far left column). In addition, the total percentage distribution of each HPLC component (M_1, M_2, D and A) in the four molecular weight fractions from G100 chromatography can be obtained by reading across the relevant row. Finally the total percentage composition (determined by HPLC) of each G100 fraction can be found be reading down the relevant column. Thus M_1 and M_2 were found in all four G100 fractions. D was found mainly in the dimer, but also in the aggregate and void fractions. A was found only in the aggregate, although there was a possible trace in the dimer.

DISCUSSION

The hGH used in this study came from a large-scale purification of human pituitaries[8]. Whilst the general experimental design was known, the precise composition of this batch was not. It was, therefore, initially reassurring to see four peaks of ir-hGH both by gel filtration chromatography and reversed-phase HPLC. As these four peaks were also present in roughly similar relative proportions in each case, the first assumption was that each peak from the gel filtration chromatogram corre-

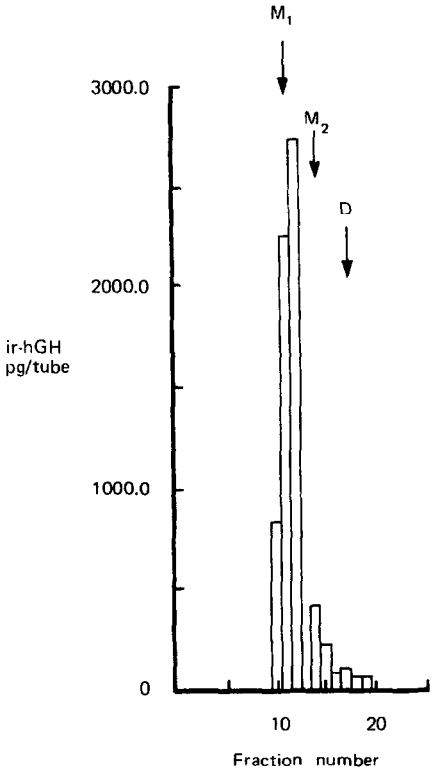


Fig. 7. Immunoreactivity profile of "void" fraction after reversed-phase HPLC. Conditions as for Fig. 4.

sponded to one from HPLC. However, when the molecular weight fractions from G100 chromatography were analysed separately by HPLC, this simplistic interpretation was proved wrong. The monomer peak (containing the bulk of ir-hGH) was further separated into two components. The more abundant M_1 almost certainly contains the 22K peptide, since this is the principal hGH form found in pituitaries and plasma, and it co-eluted with the major component in the WHO First IRP for RIA. The other component, M_2, is unknown. One possibility considered was that it is the 20K form (hGH_{22k} minus residues 32–46) since this has been reported in pituitary extracts where it comprises 5–15% of the total GH[12,13]. Subsequent work indicates that M_2 is not the 20K form, but rather a chemically modified form of hGH_{22k} (R. L. Patience, unpublished results). Consequently, the elution position of the 20K form on HPLC under these conditions remains unestablished, since no authentic standard was available; in fact the hGH used here may not contain this variant.

The dimer and aggregate fractions from G100 chromatography both gave multiple component chromatograms on reversed-phase HPLC, and the most plausible interpretation of the results is the same in both cases. Both fractions appear to contain true high molecular weight growth hormones. In the dimer fraction, D may well be the 45K interchain disulphide dimer previously identified in pituitaries[14], whilst A may be another oligomer (the separation by G100 chromatography does

TABLE I

PERCENTAGE ir-hGH FOUND IN THE VARIOUS FRACTIONS FROM G100 CHROMATOGRAPHY AND HPLC

Columns give the total percentage distribution of ir-hGH components resolved by HPLC within each molecular weight fraction from G100 chromatography. Rows show the distribution of individual HPLC components across the four molecular weight fractions.

			G100			
			Monomer	*Dimer*	*Aggregate*	*Void*
		Total	77.0	12.2	2.6	8.2
HPLC	M_1	68.7	58.4	3.1	0.8	6.4
	M_2	22.1	18.6	1.9	0.3	1.3
	D	8.0	−*	7.2	0.3	0.5
	A	1.2	−*	tr.*	1.2	−*

* tr. = trace; − = not detected.

not allow one to be more precise). However, a substantial proportion of both dimer and aggregate fractions consists of monomeric species M_1 and M_2 (and also D in the aggregate), which are presumably loosely aggregated at neutral pH, but dissociate at pH 2. It is difficult to state precisely what proportion of the dimer fraction is true dimer and what is aggregated monomer, since the (relative) immunoreactivities of the different variants in the RIA are not known. However, they are almost certainly different, as this was shown to be the case for both the interchain disulphide dimer (50% as reactive as hGH_{22k})[12] and the 20K variant (33% as reactive)[14] by previous workers in their assay.

These data for the dimer and aggregate fractions are, therefore, entirely compatible with those previously obtained for the dimer fraction in human pituitaries, only a small percentage of which is covalently bonded[14], and for the "big" (dimer) and "big–big" (aggregate) GH found in plasma[15]. This is reassurring since the analytical techniques used in these previous studies (polyacrylamide gel electrophoresis and isoelectric focusing) were quite different from the reversed-phase HPLC method used here.

Finally, the void fraction from G100 chromatography appeared to consist almost entirely of monomeric forms, with a small amount of D. No very high molecular weight form (*i.e.* $>10^5$K) appeared to be present.

These results only help to reinforce the opinion that reliance on gel filtration chromatography for analysis of hGH (whether in normal or diseased subjects) is not adequate, since each molecular weight fraction is in turn a multiple component mixture. The need for a more detailed study of the GH composition is even more apparent when comparisons are made of bioactivity and immunoreactivity.

Although the reversed-phase HPLC method described here still needs some further investigation —*e.g.* to determine the elution position of the 20K form— its ability to separate hGH variants and post-translational modifications both rapidly (less than 30 min), and with intact immunoreactivity, makes it a useful tool in the study of GH composition in normal and diseased subjects.

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