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COMPARISON OF REVERSED-PHASE AND ANION-EXCHANGE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY FOR THE ANALYSIS OF HUMAN GROWTH HORMONES

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SUMMARY

Separation of human growth hormones (hGH) has been studied by three chromatographic methods: gel filtration chromatography, reversed-phase high-performance liquid chromatography (HPLC) and anion-exchange HPLC. Six growth hormone preparations were used to characterize the systems: two pituitary extracts (an old freeze-dried purified extract A, and a freshly extracted frozen pituitary B); two purified forms (hGH_{20K} and dimer); and two chemically modified monomeric forms (reduced and deamidated).

Gel filtration chromatography (pH 8) separated the two pituitary extracts A and B into four components (monomeric, dimeric, aggregate and void material), the relative compositions of which were very similar in both extracts. Reversed-phase HPLC under acid dissociating conditions (pH 2) separated the extracts into four peaks (M_1, M_2, D and A). The first two components are both monomers: M_1 contains all those forms where no major conformational change has occurred; M_2 comprises forms with substantial conformational alteration (*e.g.* disulphide bridge cleavage). Component D includes the interchain disulphide dimer, whilst A is an uncharacterized oligomeric form.

Anion-exchange HPLC (pH 8) separated extract A into four regions of immunoreactivity. Region 1 contains hGH_{20K} separated from hGH_{22K}; region 2 includes other monomeric charge variants; region 3 is a broad peak which includes true dimers of hGH_{20K} and hGH_{22K}, as well as loosely aggregated monomer. Region 4 is another broad peak, presumably containing a higher-molecular-weight hGH form or forms.

All the hGH forms (identified and unidentified) can be separated by sequential use of two out of three chromatographic methods. No two forms found so far co-eluted in all three systems.

INTRODUCTION

The major human growth hormone (hGH) form in both the pituitary and plasma is a single chain, 191 amino acid residue peptide held in rigid conformation by two disulphide bridges (at residues 53–165 and 182–189)[1,2]. This is the so-called

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hGH_{22K} monomer with a molecular mass of 22 000 daltons. Other variants include the hGH_{20K} form (hGH_{22K} minus amino acids 32–46), cleaved monomer forms (bridge and/or chain cleavages), deamidated and acetylated variants, alkaline forms, and dimeric and possibly larger oligomers. These forms have been separated primarily, and successfully, by electrophoretic techniques[1,2], whilst chromatographic methods have been used mainly for purification[2–4].

However, high-performance liquid chromatography (HPLC) may have some advantages over electrophoresis — not necessarily in resolving power but in speed of separation and convenience of collecting fractions for further analysis(*e.g.* bioassay, immunoassay or chromatography). HPLC has been applied in a semi-preparative fashion for purification of tumour[5] and biosynthetic[6] hGH, whilst one previous study[7] of hGH variants demonstrated the difficulty in separating the 22K and 20K monomers. This paper is the second part of our study of HPLC separation modes for analysis of hGH variants. In the first part[8], a freeze-dried, partially purified pituitary extract was examined by conventional gel filtration chromatography and reversed-phase HPLC on a C_3 wide-pore column. In the present work, these results are compared with those obtained by the same techniques for a freshly extracted frozen pituitary. In addition, results from reversed-phase HPLC for the freeze-dried pituitary extract are contrasted with data from weak anion-exchange HPLC. Finally, the elution positions of certain variants have been established with the aid of material purified by electrophoresis, and with chemically modified monomeric hGH.

EXPERIMENTAL

Pituitary hGH

The pituitary hGH was derived from two sources: partially purified, freeze-dried hGH (extract A) and freshly extracted frozen pituitary (extract B). Extract A came from a large-scale purification[4], which involved separation by gel filtration chromatography and anion exchange chromatography. The purified material had been freeze-dried and stored at −20°C for several years prior to this study. Extract B came from a pituitary (189 mg, wet weight), which had been stored at −20°C for *ca.* 6 months prior to extraction. It was then homogenized (1 min) in 2 ml of a cold saline solution (0.154 *M* sodium chloride–0.05 *M* phosphate buffer; pH 7.4) containing 15 m*M* sodium azide and 0.15 m*M* phenylmethylsulphonyl fluoride. This was centrifuged at 1700 *g* for 30 min (4°C) and the supernatant was decanted and aliquoted into 0.25-ml portions. These were stored at −70°C until used.

Purified growth hormone forms

The 20K form, and a fraction containing covalently-bonded dimeric hGH (and some monomer material), were gifts from Professor U. J. Lewis, and had been purified as described previously[9,10].

Gel filtration chromatography

Gel filtration chromatography was carried out on a column (100 × 1.5 cm I.D.) of Sephadex G-100 superfine (Pharmacia) eluted with 0.05 *M* ammonium bicarbonate–0.1% (w/v) human serum albumin (HSA)–15 m*M* sodium azide buffer (pH 8.0) at *ca.* 3 ml/h. Fractions were collected for 40 min each (*ca.* 2 ml). The void

volume was marked with bovine thyroglobulin (669 000 daltons mol. wt.) and the salt peak by potassium iodide (visualized by addition of acidified hydrogen peroxide). Typically, hGH (200 ng) was loaded in 0.5–1.0 ml of buffer.

High-performance liquid chromatography

HPLC was performed on a Varian 5560 fitted with a Rheodyne 7125 loop injector. Detection was at 280 nm and the signal was recorded on a dual pen chart recorder (JJ Instruments). Fractions (1.06 ml) were collected with a Frac 300 (Pharmacia) into plastic tubes containing either 1 ml of 0.1% (v/v) Triton X-100 aqueous solution, or 0.05 ml of 2% Triton X-100 or 10% (w/v) bovine serum albumin (BSA), to prevent hormone adsorption to the tube.

Reversed-phase HPLC was carried out using an Ultrapore RPSC column (75 × 4.6 mm I.D.) (Altex). The solvents were water–0.1% (v/v) trifluoroacetic acid (TFA) (solvent A; pH 2) and 1-propanol–0.1% (v/v) TFA (solvent B). Typically, the growth hormones were eluted using a gradient of 26–35% B over 27 min at a flow-rate of 1 ml/min; although exact initial and final conditions varied with analyses, gradient slope (0.33% B per min) remained constant.

For anion-exchange HPLC, a TSK DEAE 5-PW column (75 × 7.5 mm I.D.) (Toyo Soda) was used. Solvents were 0.05 *M* ammonium acetate (solvent A) and 0.5 *M* ammonium bicarbonate in A (solvent B). The gradient elution was from 0 to 100% B in 60 min at a flow-rate of 1 ml/min. For all HPLC analyses, peak identification was made by co-injection or comparison of retention volumes of "standard" hGH preparations run on the same day and under the same conditions.

Reagents

Water was deionized, 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 G-100 chromatography and HPLC were quantified by radioimmunoassay (RIA). Fractions were either dried by vacuum desiccation over self-indicating silica gel and sodium hydroxide pellets, or assayed directly. Dried fractions were reconstituted in assay buffer [0.05 *M* phosphate (pH 7.4)–1% (w/v) BSA or 2% (v/v) horse serum–15 m*M* sodium azide]. A 50-μl aliquot of each fraction was diluted to 400 μl with assay buffer, and 50 μl of sheep antibody (initial dilution 1:92 000) were added. After a 24-h incubation, 50 μl of [^{125}I]hGH (*ca.* 12.5 nCi) 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 anti-sheep) and centrifugation. Standards were prepared from the WHO 1st International Reference Preparation (IRP) (66/217) by dilution in 0.05 *M* phosphate buffer–0.5% (w/v) HSA or 1% (w/v) BSA–15 m*M* sodium azide (pH 7.4) and stored at −20°C.

Chemical modifications of monomeric hGH

Monomeric hGH was obtained from gel filtration chromatography of the WHO 1st IRP for RIA and subjected to chemical reduction and deamidation. The

dried hGH (*ca.* 1.5 μg) was reconstituted to 2 ml in 0.05 *M* ammonium bicarbonate–0.1% (w/v) HSA–0.1% (w/v) sodium azide.

Reduction. To this solution (0.25 ml; 187.5 ng of hGH) were added 5 μl (64 nmol) of 2-mercaptoethanol. This was left at room temperature for 4 h[10].

Deamidation. The same solution (1 ml; 750 ng of hGH) was adjusted to pH 9 with ammonium hydroxide and incubated at 37°C for 70 h (based on the method of Lewis *et al.*[11]).

At the end of reduction and deamidation, the modified monomeric hGH was analysed by reversed-phase and anion exchange HPLC.

RESULTS

Three different chromatographic methods were used to study six different growth hormone preparations. The two pituitary extracts were analysed by gel filtration chromatography and then reversed-phase (extracts A and B) and anion-exchange (extract A only) HPLC of the various molecular weight fractions.

The 20K standard, reduced and deamidated monomeric hGHs were analysed by reversed-phase and anion-exchange HPLC. The dimeric standard material was analysed by reversed-phase HPLC only. All figures quoted refer to percentage of the total immunoreactivity recovered.

Pituitary extracts

Gel filtration chromatography. For extracts A and B, four peaks of immunoreactivity were obtained (Fig. 1). The major peak (77.0% of recovered immunoreactivity for A and 77.0% for B) co-eluted with the major peak of the WHO 1st IRP for RIA, and with radiolabelled monomeric hGH, and was thus deemed to contain the monomeric forms, including the principal hGH_{22K} and hGH_{20K}. Three other discrete peaks are also present: 'dimer' (K_{av} value *ca.* half that of the monomer) (12.2% for A and 13.5% for B); "aggregate" ($K_{av} \simeq 1/3\ K_{av}$ monomer) (2.6% for A and 5.2% for B); "void" material (8.2% for A and 4.3% for B). The relative percentage compositions of each fraction are shown in Table I.

The fractions constituting each of the four peaks were then combined and analysed by reversed-phase HPLC (extracts A and B) and anion-exchange HPLC (extract A).

Reversed-phase HPLC. The two extracts were analysed under conditions that have been shown previously[8] to separate extract A into four components — two monomeric, one dimeric and one other oligomer. The results for extract A are shown again here for direct comparison with extract B.

The monomer fraction from gel filtration chromatography of extract B was further resolved by reversed-phase HPLC (Fig. 2b) into one principal component M_1 and a very minor peak M_2. This contrasts with extract A, where M_2 constituted one-quarter of the recovered immunoreactivity (Fig. 2a). For the dimer fraction of extract B, M_1 was again the dominant form, although there was a significant peak co-eluting with D (Fig. 3b). This is substantially different to the profile for extract A (Fig. 3a), where D was the major form present. Similarly, the aggregate fraction for extract B contained mainly M_1 with small amounts of M_2, D and A (Fig. 4b), whilst extract A had M_1 and A as the two principal peaks (Fig. 4a). The void-volume

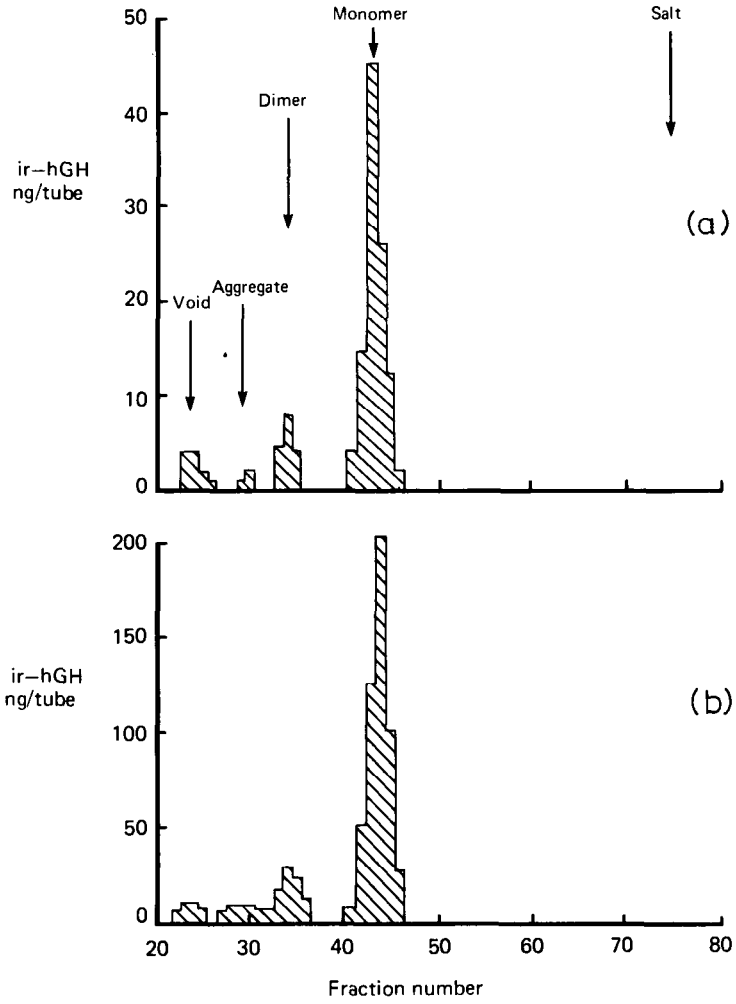

Fig. 1. Immunoreactive hGH profiles of two pituitary extracts after gel filtration chromatography on Sephadex G-100. (a) Freeze-dried purified extract A; (b) extracted frozen pituitary B.

material of both extracts contained mostly monomeric forms, primarily M_1 (78% for A and 60% for B) and some M_2.

In addition to calculating the relative amounts of M_1, M_2, D and A within each molecular weight fraction, the relative proportions of each of these four forms were calculated for the extracts as a whole. These are given in Table I. Thus, M_1 was found to constitute *ca.* 68.7% of extract A and 87.0% of B, whilst M_2 was more abundant in extract A (22.1%) than extract B (6.3%). The relative amounts of D and A were low in both cases (<8%).

Anion-exchange HPLC. Extract A was chromatographed first and produced a complex profile that contained four regions of immunoreactivity (1–4). Individual

TABLE I

COMPARISON OF GEL FILTRATION CHROMATOGRAPHY AND HPLC FOR DETERMINING THE IMMUNOREACTIVE (ir) GROWTH HORMONE COMPOSITION (%) OF TWO PITUITARY EXTRACTS

Separation method	*ir-hGH peak*	*Pituitary extract ir-hGH*	
		A	*B*
Gel filtration chromatography	Monomer	77.0	77.0
	Dimer	12.2	13.5
	Aggregate	2.6	5.2
	Void	8.2	4.3
Reversed-phase HPLC	M_1	68.7	87.0
	M_2	22.1	6.3
	D	8.0	3.0
	A	1.2	2.0
	Pre-M_1	–	1.7
Anion-exchange HPLC	M_A	4.7	
	M_B	40.3	
	M_C	22.1	n.a.*
	M_D	13.8	
	Dimer	8.8	
	Aggregate	2.1	
	Unaccounted**	8.2	

* n.a. = Not analysed.
** Unaccounted = material eluting in gel filtration void volume.

molecular weight fractions from gel filtration chromatography were also analysed, in an identical manner to those subjected to reversed-phase HPLC. The monomer fraction (Fig. 5a) produced four peaks of immunoreactivity, labelled M_A, M_B, M_C and M_D. M_A and M_B constituted region 1, whilst M_C and M_D formed region 2. The dimer fraction (Fig. 5b) (region 3) contained one very broad peak covering approximately eleven fractions, and small amounts of M_B, M_C and possibly M_D on the leading edge of the dimer peak. In contrast, the aggregate fraction (region 4) (Fig. 5c) contained one, perhaps two, peak(s), eluting after the broad peak in the dimer fraction.

The material eluting in the void by gel filtration chromatography produced no discrete peaks of immunoreactivity by anion-exchange HPLC.

The relative proportions of the various peaks (M_A to M_D, dimer, aggregate) for the extract as a whole were calculated, as they were for reversed-phase HPLC, and these data are also shown in Table I. The major peak was M_B (40.3%), with sizeable amounts of M_C (22.1%) and M_D (13.8%) and a small amount of M_A (4.7%). The higher-molecular-weight forms comprised *ca.* 11% of the total, whilst *ca.* 8% was unassigned (the material eluting in the void of the gel filtration chromatographic analysis).

Purified hGH_{20K} and dimer

Reversed-phase HPLC. Almost half of the immunoreactivity for the 20K ma-

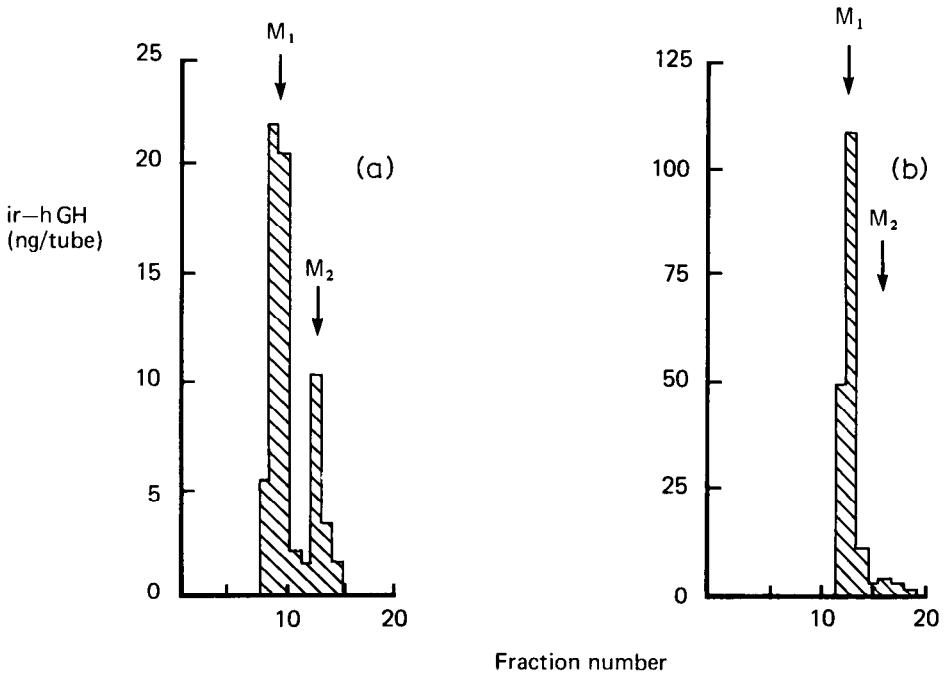

Fig. 2. Immunoreactive hGH profiles after reversed-phase HPLC of the monomer fractions from gel filtration chromatography of (a) extract A and (b) extract B. Conditions: Altex Ultrapore RPSC column eluted with a typical gradient of 26–35% B in 27 min at a flow-rate of 1 ml/min. Solvent A: 0.1% (v/v) aqueous TFA. Solvent B: 0.1% (v/v) TFA in 1-propanol, 1.06-ml fractions collected into Triton X-100 and/or BSA and assayed by RIA.

terial co-eluted with component M_1. Of the remainder, 25% eluted with component D, and the rest as two later eluting peaks. The dimer gave four virtually equal peaks, eluting with components M_1, M_2, D and A.

Anion-exchange HPLC. The 20K standard produced three peaks of immunoreactivity: one peak co-eluting with M_A; a second, very minor component, which eluted between M_B and M_C; and a third peak, which eluted in the same position as the dimer in Fig. 5b.

Deamidated and reduced monomeric hGH

Reversed-phase HPLC. The unmodified monomer was resolved into three components. The major component co-eluted with M_1, with small amounts eluting with M_2 and before M_1 (pre-M_1). After deamidation, the relative proportions of these three forms were effectively unchanged (Table IIa). In contrast, after reduction there was an increase in the amount of M_2 at the expense of M_1, with pre-M_1 unaltered (Table IIa).

Anion-exchange HPLC. The original monomer produced only two virtually equal peaks of immunoreactivity, co-eluting with M_B and M_C (Table IIb). However, after deamidation, M_B had effectively disappeared, and had been replaced by a major peak co-eluting with M_D (Table IIb). The relative proportion of M_C also declined

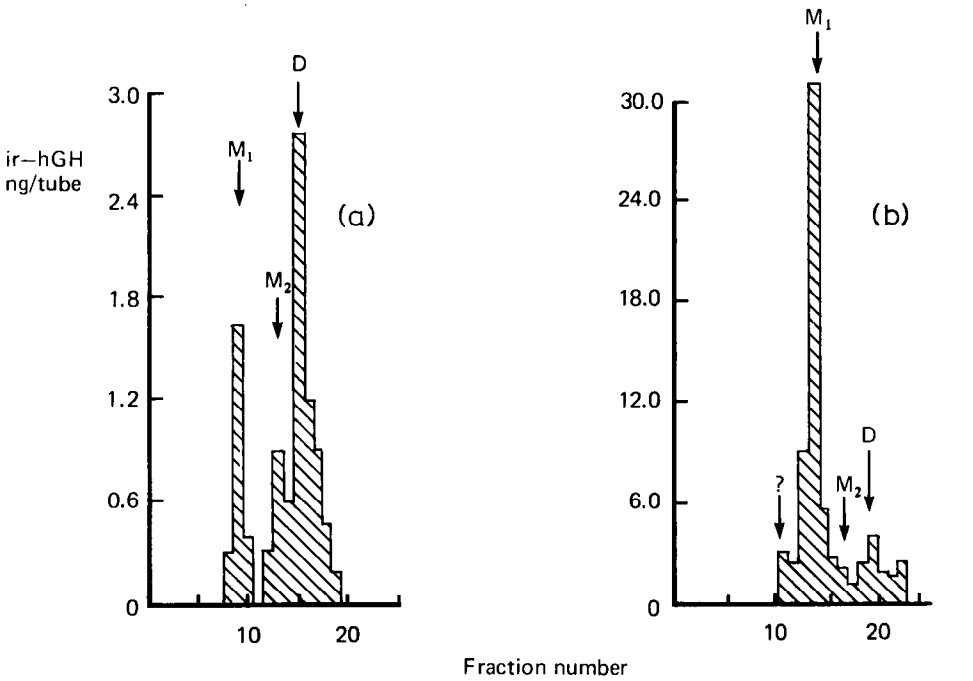

Fig. 3. Immunoreactive hGH profiles after reversed-phase HPLC of the dimer fractions from gel filtration chromatography of (a) extract A and (b) extract B. Conditions as in Fig. 2.

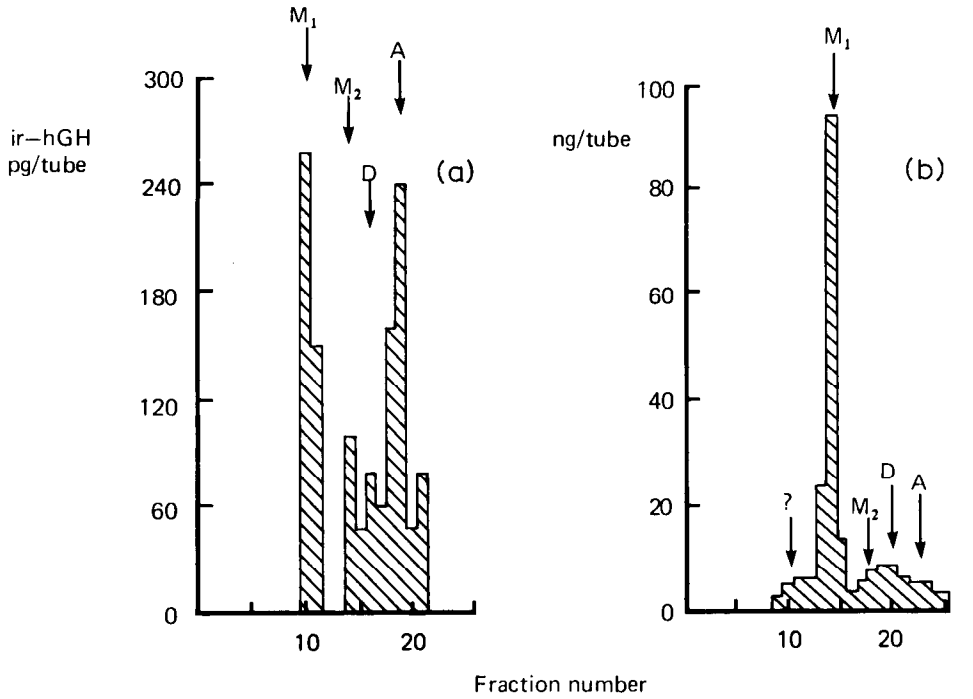

Fig. 4. Immunoreactive hGH profiles after reversed-phase HPLC of the aggregate fraction from gel filtration chromatography of (a) extract A and (b) extract B. Conditions as in Fig. 2.

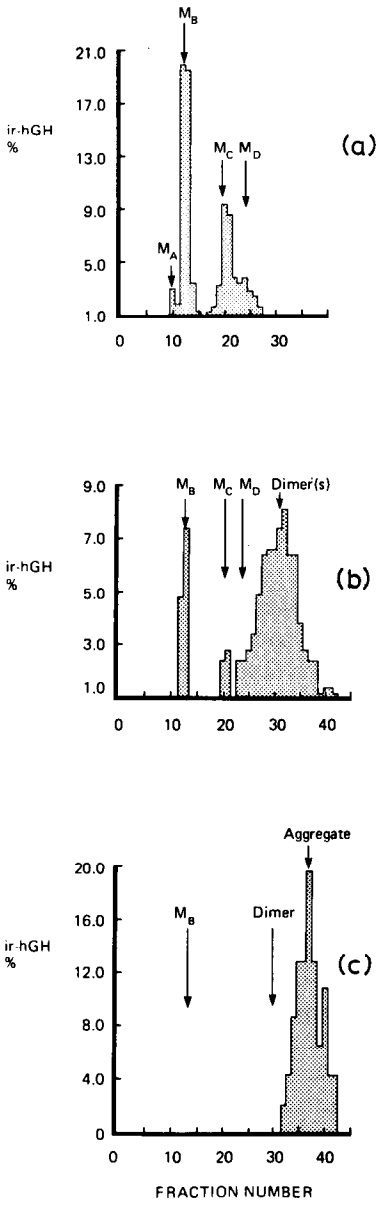

Fig. 5. Immunoreactive hGH profiles after anion-exchange HPLC of the (a) monomer, (b) dimer and (c) aggregate fractions from gel filtration chromatography of extract A. Conditions: TSK DEAE 5-PW column eluted with a gradient of 0–100% B in 60 min. Solvent A: 0.05 *M* ammonium acetate. Solvent B: 0.5 *M* ammonium bicarbonate in solvent A. Fractions (1.06 ml) collected into Triton X-100 and/or BSA and assayed by RIA.

slightly, although this may not be a significant decrease but simply assay variation. The reduced monomer showed a decline in M_B and M_C, with a corresponding rise in a peak co-eluting with M_D not previously detected (Table IIb).

TABLE II

ANALYSIS OF DEAMIDATED AND REDUCED hGH MONOMER BY REVERSED-PHASE HPLC AND BY ANION-EXCHANGE HPLC

ir-hGH peak	*ir-hGH (%)*		
	Monomer	*Deamidated*	*Reduced*
(a) Reversed-phase HPLC			
M_1	84.0	83.4	63.3
M_2	8.8	9.3	29.2
pre-M_1	7.2	7.3	7.5
(b) Anion-exchange HPLC			
M_B	46.8	3.5	36.6
M_C	53.2	44.9	46.2
M_D	–	45.7	17.2
pre-M_A	–	5.8	–

DISCUSSION

The purpose of this study was, primarily, to establish HPLC methods that could be used to separate the known hGH forms. The work so far has been essentially qualitative, *i.e.* what elutes where? Establishing elution positions is made difficult by the absence of synthetic standards and a shortage of highly purified forms of biological origin. Consequently, a number of preparations were used (two pituitary extracts, two purified individual variants and two chemical modifications) to aid characterization of the two HPLC systems.

The two pituitary extracts (A and B) produced virtually identical profiles of hGH immunoreactivity after gel filtration chromatography. However, after reversed-phase HPLC, a number of striking differences were observed, both between the two techniques and between the two extracts. When results for the two extracts were compared, the relative amount of M_2 was far higher in extract A than in B, and may therefore be a storage artefact. The higher-molecular-weight fractions of extract A (dimer and aggregate) appeared to contain true higher-molecular-weight forms (D in the dimer and A in the aggregate fractions), but also a proportion of monomeric forms (*ca.* 40% of recovered immunoreactivity). In contrast, extract B had a far greater proportion of monomer in both dimer (66%) and aggregate (76%) fractions. In both extracts, the void material consisted almost entirely of monomeric forms. It is known from gel electrophoresis[9] that higher-molecular-weight fractions from gel filtration chromatography of pituitary extracts contain mainly loosely aggregated monomer, and very little covalently bonded dimer of the other oligomer. Presumably, the acidic dissociating conditions used with reversed-phase HPLC disaggregated the monomer in the oligomer fractions, in a similar fashion to gel electrophoresis under reducing conditions. As a result, reversed-phase HPLC gave a higher relative proportion of monomeric forms in the extracts than did gel filtration chromatography.

To corroborate and extend these conclusions, assignment was made of elution positions of certain key hGH forms. Hence, purified hGH_{20K}, dimer, reduced and deamidated monomers, were analysed separately by reversed-phase HPLC. The pur-

ified 20K form contained a major peak eluting with M_1, and three components eluting with D, A and after A. The conclusion to be drawn is that monomeric hGH_{20K} co-elutes with M_1; the other components are probably dimer, and another oligomer, forms of hGH_{20K}, since this component is known to aggregate readily (U. J. Lewis, personal communication; and ref. 10), and the sample had been stored as a frozen solution at −20°C for *ca.* two weeks prior to analysis. Deamidated monomeric hGH showed exactly the same immunoreactivity profile as the untreated monomer, although anion-exchange HPLC showed that a chemical change had occurred (discussed later). Consequently, the deamidated form also elutes with M_1. In contrast, 2-merceptoethanol-reduced monomer eluted with M_2. This is presumably because reduction cleaves the disulphide bridge(s)[9], opening up the molecule and altering its conformation. This exposes different regions of the molecule to the reversed-phase packing material and, in this case, increases the retention time. The chromatogram for the purified dimer showed peaks eluting with M_1, M_2, D and A. This material was known to contain monomeric forms (U. J. Lewis, personal communication), which eluted as M_1 and M_2. The true dimer presumably eluted as component D.

To summarize these results for reversed-phase HPLC: component M_1 contains any monomeric hGH where the overall confirmation of the molecule is retained. This includes hGH_{22K}, hGH_{20K}, deamidated hGH and also [^{125}I]hGH. Component M_2 contains monomer forms where the conformation has been substantially altered, *e.g.* by cleavage of one or more disulphide bridges. Component D contains covalently-bonded dimers of both hGH_{22K} and hGH_{20K}. Component A is unknown but may contain "cleaved" forms of hGH dimer and/or higher-molecular-weight forms.

The potential of anion-exchange HPLC was also investigated, using the same general approach as for characterization of the reversed-phase HPLC system. In this case, only pituitary extract A and its molecular weight fractions were analysed, along with the two chemical modifications and the purified 20K form. When extract A was chromatographed, the resulting immunoreactivity profile was rather complex and could only be classified into four general regions 1–4. However, analysis of the molecular weight fractions from gel filtration chromatography, and the three monomer preparations, permitted interpretation of the profile.

The monomer fraction of extract A contained a major component labelled M_B in region 1. As M_B was also the major component in the monomer fraction of the WHO 1st IRP for RIA, it appears that this is the principal hGH_{22K} form. The other component in region 1 is M_A. This co-eluted with the major peak after analysis of the 20K material. Consequently, this system appears to resolve monomeric hGH_{22K} and hGH_{20K}. The monomeric fraction of extract A also contained a second region of immunoreactivity, with peaks labelled M_C and M_D. Interestingly, the two chemical modifications (deamidation and reduced) both co-eluted with M_D (Table IIb), and consequently M_C remains uncharacterized. In view of the latter's relative abundance (see Table I), M_C almost certainly contains component(s) which elute as M_2 in the reversed-phase HPLC system. However, although chemically reduced monomer eluted with M_2 (and was the only component so far found to do so), it does not elute with M_C. It is possible that M_C may be a chain-cleaved form(s) where the bridges remain intact, since this has been found in pituitary extracts[1], although there is no direct evidence to support this hypothesis. It is also interesting to note that monomeric [^{125}I]hGH, which co-eluted with M_1 under reversed-phase HPLC, co-eluted

principally with M_D after anion exchange HPLC (R. L. Patience, unpublished results). This would imply that some chemical change has occurred on iodination, which was not detected by reversed-phase HPLC, although whether this is simply the introduction of iodine atom(s), or an additional modification, cannot be resolved at present. It can be concluded, however, that the monomeric hGH forms eluting in region 2 include all those that can be classified as charge variants of hGH_{22K}. The only exception is hGH_{20K}, where deletion of residues 32–46 appears to have less effect on retention.

The dimer fraction from extract A produced a single, broad peak by anion exchange HPLC (region 3), eluting after M_D. There was a small amount of monomeric material also present, but far less than after reversed-phase HPLC. This is presumably because the anion exchange HPLC system was operated at pH 7.5–8.0 and disaggregation scarcely took place. The width of the peak may be a product of either poor chromatography or the presence of a multiple component mixture. The latter seems more probable, since the sample could well contain covalently-bonded dimeric hGH_{22K}, hGH_{20K} (the purified material gave a peak in this region) and loosely aggregated dimer(s). The aggregate fraction gave a very similar result, with one broad peak eluting in region 4. In this case, no monomer material was observed. The void material, of which there was very little, gave no discrete peaks of immunoreactivity after analysis. This is the material "unaccounted" for in the summary of results in Table I. The elution order of the hGH forms on anion exchange HPLC is in accordance with available information on p*I* values, and with elution orders on DEAE-cellulose. It was shown by isoelectric focusing that hGH_{22K} had a p*I* of 5.6 whereas for hGH_{20K} it was 5.85[10]. As the pH of the mobile phase was *ca.* 8.0, one would expect the slightly less acidic form to elute slightly before the main 22K monomer, as observed. The deamidated, and therefore more acidic, form elutes later than hGH_{22K} both on HPLC and on DEAE-cellulose[11], whilst the elution position of the dimer can also be explained in terms of its greater negative charge[9] than the 22K form. Although elution position can be justified in this case on the basis of net anionic charge, it is unlikely that separation will be based entirely on this one mechanism[12].

To summarize, of all the hGH forms characterized to date, no two elute in the same position in all three systems. In other words, any two components that co-elute in two systems (*e.g.* hGH_{22K} and hGH_{20K} by gel filtration chromatography and reversed-phase HPLC) can be separated on the third system (*e.g.* anion-exchange HPLC). Used in conjunction, these three techniques appear to offer potential for characterizing hGH in endocrine samples.

The next stage of the work will be to make the methods more rigorously quantitative. However, typical recoveries can be illustrated using the WHO 1st IRP for RIA. By reversed-phase HPLC, recoveries were 78 and 85%, whilst anion-exchange HPLC gave 84 and 93% of loaded immunoreactivity for repeat analyses. However, if fractions are then dried by vacuum desiccation, recoveries can drop substantially [36% (n = 3) and 74% (n = 3) for hGH in reversed-phase and anion-exchange HPLC solvents, respectively]. Therefore, all fractions (including those consisting of aqueous organic solvents) are now assayed by RIA immediately after collection, wherever possible.

This study demonstrates the applicability of this approach to other hormones (such as prolactin) that exist in a variety of immunoreactive and bioactive forms.

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REFERENCES

1 U. J. Lewis, R. N. P. Singh, M. B. Sigel, L. G. Frigeri, Y. N. Sinha and W. P. VanderLaan, in B. B. Saxena, K. J. Catt, L. Birnbauer and L. Martini (Editors), *Hormone Receptors in Growth and Reproduction*, Raven Press, New York, 1984, p. 37.
2 G. Baumann, J. G. MacCart and K. Amburn, *J. Clin. Endocrinol. Metab.*, 56 (1983) 946.
3 R. N. P. Singh, B. K. Seavy and U. J. Lewis, *Endocrinol. Res. Commun.*, 1 (1974) 449.
4 R. Lumley Jones, G. Benker, P. R. Salacinski, T. J. Lloyd and P. J. Lowry, *J. Endocrinol.*, 82 (1979) 77.
5 W.-C. Chang, A. L.-Y. Chen, C.-K. Chou and L.-T. Ho, *Int. J. Pept. Protein Res.*, 23 (1984) 637.
6 W. J. Kohr, R. Keck and R. N. Harkins, *Anal. Biochem.*, 122 (1982) 348.
7 B. Grego and M. T. W. Hearn, *J. Chromatogr.*, 336 (1984) 25.
8 R. L. Patience and L. H. Rees, *J. Chromatogr.*, 324 (1985) 385.
9 U. J. Lewis, S. M. Peterson, L. F. Bonewald, B. K. Seavey and W. P. VanderLaan, *J. Biol. Chem.*, 252 (1977) 3697.
10 U. J. Lewis, J. T. Dunn, L. F. Bonewald, B. K. Seavey and W. P. VanderLaan, *J. Biol. Chem.*, 253 (1978) 2697.
11 U. J. Lewis, R. N. P. Singh, L. F. Bonewald and B. K. Seavey, *J. Biol. Chem.*, 256 (1981) 11645.
12 W. Kopaciewicz and F. E. Regnier, *Anal. Biochem.*, 133 (1983) 251.