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Note

Reversed-phase high-performance liquid chromatography of human growth hormone*

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Numerous studies have demonstrated that reversed-phase high-performance liquid chromatography (RP-HPLC) is an extremely powerful technique for polypeptide analysis. However, with increasing molecular weight and/or hydrophobicity, the applicability of the method seems to be seriously reduced. For human growth hormone (hGH), a 22-kilodalton (22 k) hydrophobic protein with a well known amino acid sequence, the limited number of published RP-HPLC analyses reflects these difficulties.

After it had been demonstrated that hGH could be eluted from a reversed-phase column using known mobile phases[1–4], RP-HPLC analyses were used as identification criteria for tumour-derived hGH[5], hGH purified by preparative high-performance ion-exchange chromatography[6], biosynthetic methionyl-hGH[7] and biosynthetic hGH[8].

The peptides derived from a tryptic digest of hGH have been succesfully separated[3,5,8–12], primarily using trifluoroacetic acid (TFA)–acetonitrile as the mobile phase and C_{18} stationary phases. Three reports have described the separation between 22k hGH and hGH-related substances: an incipient separation between 20k and 22k hGH[13] and the separation of pituitary-derived hGH in four fractions[14,15]. One of these fractions contained monomeric 22k and 20k hGH, deamidated hGH and iodinated hGH, and this fraction was separated from hGH with one or more S–S bonds being cleaved, hGH dimers and (probably) "cleaved" dimer or higher aggregates[15].

An ideal RP-HPLC analysis of hGH should be able to separate 22k hGH from all hGH-related substances, such as 20k and 24k hGH, deaminated hGH, partially cleaved hGHs, methionyl (met)-hGH and further extended forms of hGH. Although

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several stationary phases have been used, the limited separation capacity of the published RP-HPLC analyses may be due to the widespread use of TFA as the mobile phase. In contrast to RP-HPLC analyses of the smaller peptides derived from tryptic digests of hGH, for which TFA–acetonitrile seems to be the optimal mobile phase, other buffer–ion-pairing substances may be needed in order to increase the selectivity and separation capacity in reversed-phase hGH analyses.

We have investigated the influence of numerous buffer–modifier compositions on the reversed-phase separation of hGH preparations using mainly a silica-based and a resin-based stationary phase. In this paper we present a number of optimized RP-HPLC analyses, with high recoveries, resulting in the separation of 22k hGH from most of the above-mentioned hGH-related substances.

EXPERIMENTAL

HPLC equipment

Waters M6000 pumps, a WISP 710B, a Model 660 solvent programmer, a 720 system controller, a 730 data module and a 840 chromatography control station were used. Waters M480 and M490, a Hitachi 655A and a Pye Unicam UV photometer were employed.

Silica-based RP-HPLC

The following columns were used: Nucleosil C_{18}, 7 μm (250 × 4.0 mm I.D.), with pore size 120, 300, 500 or 1000 Å; and Nucleosil C_4, 7 μm (250 × 4.0 mm I.D.), with pore size 1000 Å.

The mobile phases were (A) buffer–(B) 90% aqueous acetonitrile, the buffer (A) being (1) 0.225 *M* $(NH_4)_2SO_4$–0.09 *M* NaH_2PO_4 (pH 2.5, adjusted with $HClO_4$); and (2) 0.200 *M* $(NH_4)_2HPO_4$ (pH 6.5, adjusted with H_3PO_4), with a linear gradient from 52 to 56% B during the 60 min. The flow-rate was (1) 1.0 or (2) or 0.5 ml/min. The temperature was 45°C.

Resin-based RP-HPLC

The following columns were used: TSK Phenyl 5PW RP, 10 μm (75 × 7.5 mm I.D.) and PLRP-S, 5 μm (100 Å) or 7 μm (300 Å) (150 × 4.6 mm I.D.).

The mobile phases were (A) buffer–(B) 90% aqueous acetonitrile, the buffers (A) being as follows: (3) 0.200 *M* $(NH_4)_2HPO_4$ (pH 7.0, adjusted with H_3PO_4); and (4) 0.250 *M* triethylammonium phosphate (pH 5.0). Two linear gradients were used: from 25 to 50% B during 60 min at ambient temperature with a flow-rate of 0.3 ml/min (phGH analyses), and from 40 to 50% B during 60 min at 45°C with a flow-rate of 0.3 ml/min (met-/22k hGH analyses).

Samples

Pituitary-derived hGH (phGH), biosynthetic hGH (bhGH) and biosynthetic methionyl-hGH (met-hGH) were obtained from Nordisk Gentofte A/S (Gentofte, Denmark)

Recovery

The area under the UV curve after gradient elution relative to that obtained after injecting the same sample directly into the UV photometer, or comparison between the area under the UV curves obtained after having collected and re-injected the eluted sample four times, were measured.

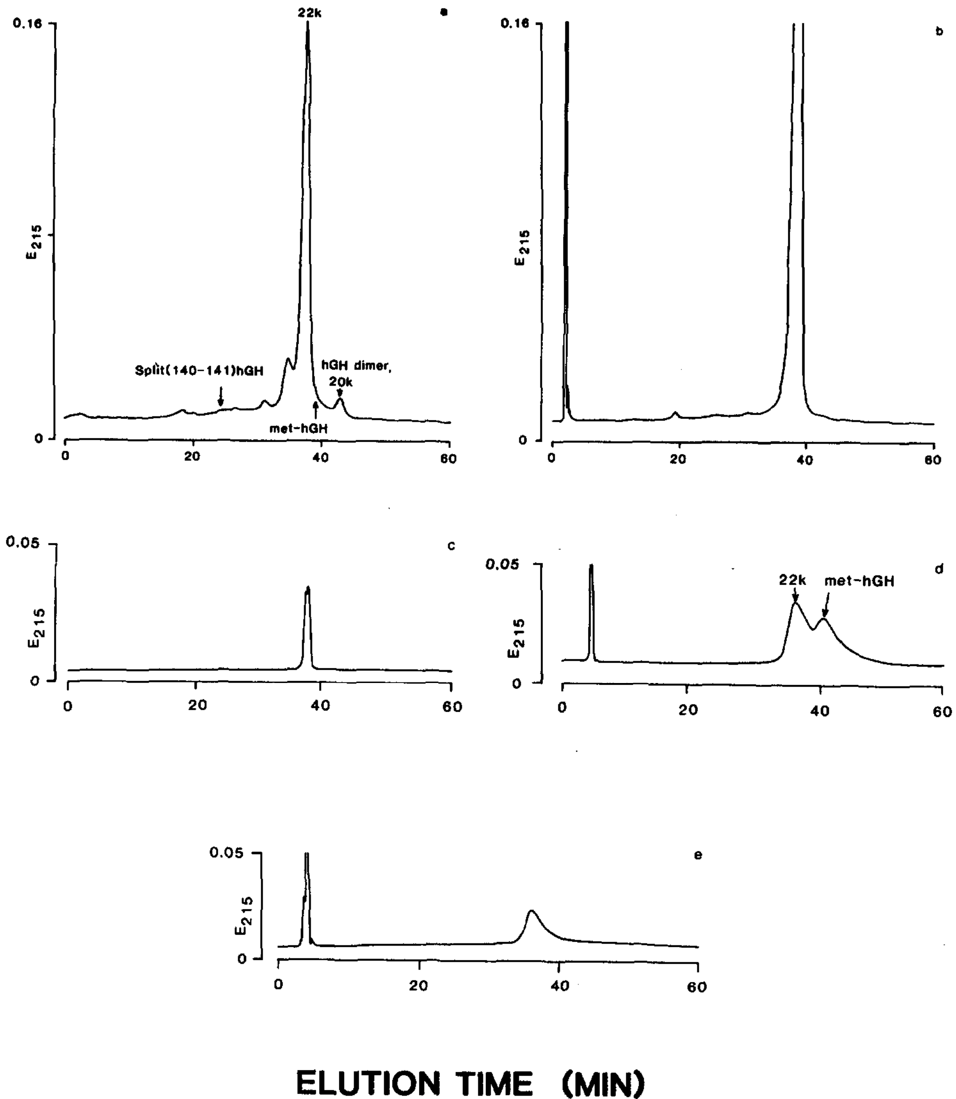

Fig. 1. Silica-based RP-HPLC separations of hGH. Column: Nucleosil C_{18} (7 μm), 300 Å. Samples: 20 μg phGH (a), 40 μg bhGH (b), 5 μg met-hGH–22k hGH mixture (c and d), 2 μg phGH (e). Mobile phases: $(NH_4)_2HPO_4$–NaH_2PO_4 (pH 2.5, $HClO_4$) (a, b and c); $(NH_4)_2HPO_4$–H_3PO_4 (pH 6.5) (d and e).

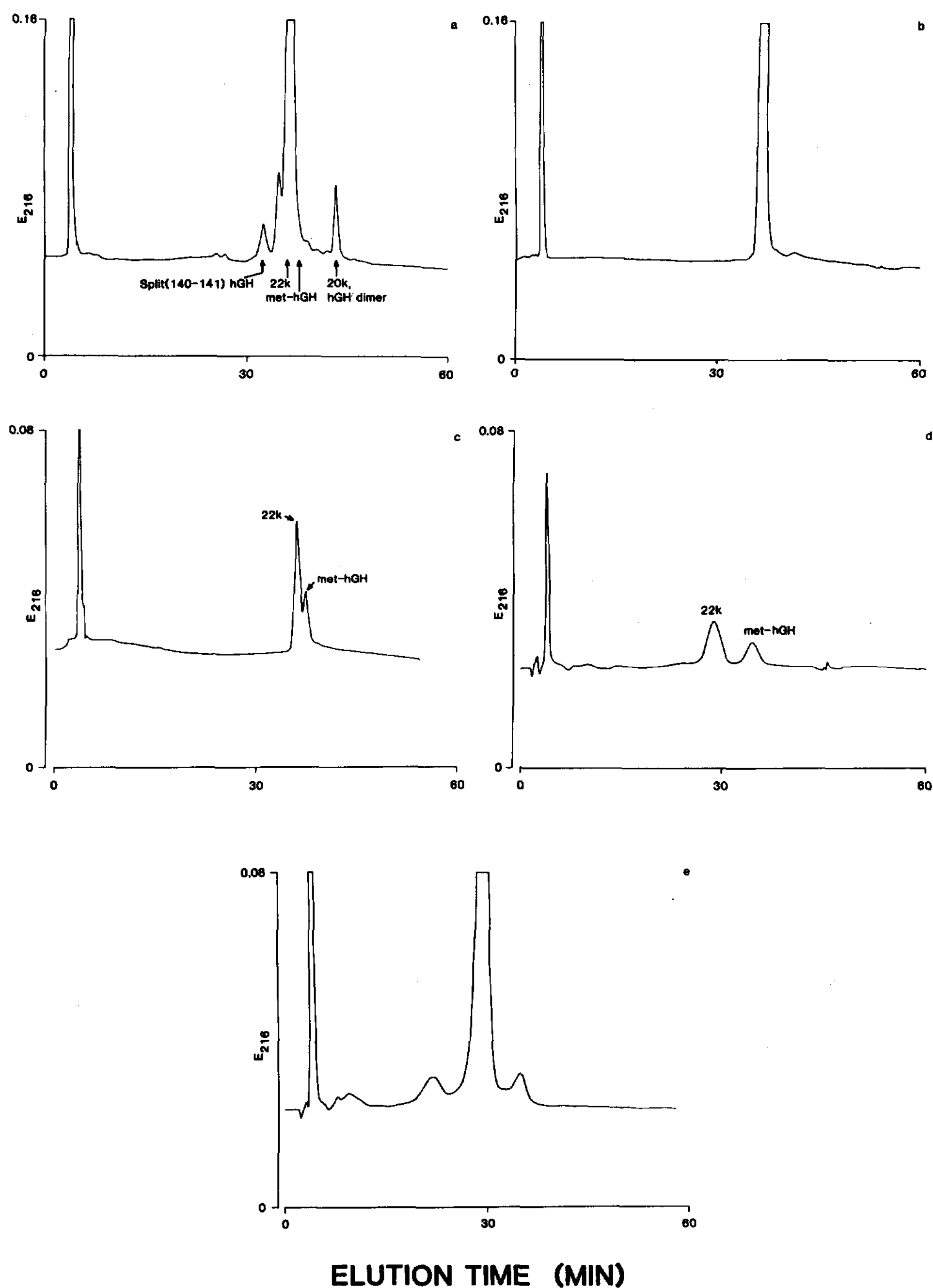

Fig. 2. Resin-based RP-HPLC separations of hGH. Column: TSK Phenyl 5PW RP. Samples: 20 μg phGH (a), 20 μg bhGH (b), 5 μg met-hGH/22k hGH mixture (c and d), 20 μg phGH (e). Mobile phase: $(NH_4)_2HPO_4$–H_3PO_4 (pH 7.0) (A)–90% aqueous acetonitrile (B). Gradients: 25 to 50% B, room temperature (a, b and c); 40 to 50% B, 45°C (d and e).

RESULTS

The separation of phGH, of bhGH and of a mixture of met-hGH and 22k hGH are shown in Fig. 1 (silica-based stationary phase) and Fig. 2 (resin-based stationary phase).

In both systems phGH was found to be heterogeneous: in addition to 22k hGH, which was the most abundant constituent (70–80%), 10–12 additional components were resolved from the main peak. A number of authentic hGH-related substances were well separated from 22k hGH in both systems (Figs. 1a and 2a). All components separated were found to react with polyclonal anti-phGH antibodies (data not shown). In contrast, bhGH was found to be almost homogeneous: 22k hGH constituted more than 98% of the protein present (Figs. 1b and 2b).

Under these conditions, however, only an inadequate separation between 22k hGH and met-hGH was obtained (Figs. 1c and 2c). In both the resin-based and the silica-based systems, the optimal separation between 22k hGH and met-hGH was obtained at neutral pH, at elevated temperature (45°C) and eluting with a shallower gradient (0.1–0.2% acetonitrile per minute) (see Figs. 1d and 2d).

If phGH was analysed under these conditions, the detailed separation pattern obtained in Figs. 1a and 2a seriously deteriorated (Figs. 1e and 2e).

For the resin-based stationary phase, the separation of phGH and that of met-hGH and 22k hGH could be obtained equally well at pH 7 and 8, whereas both the recovery and the separation capacity were seriously reduced at pH 9, using buffer system 3. When triethylammonium phosphate buffer at pH 5 was used as the eluent, only a partial separation between met-hGH and 22k hGH was obtained, and the separation capacity of phGH was less satisfasctory than that obtained using ammonium phosphate at pH 7 or 8. No effect was obtained on adding 0.1 *M* sodium perchorate to buffer system 3 (at pH 7 and 8) (data not shown).

For the silica-based stationary phase, the phGH separation at acidic pH (Fig. 1a) was optimal only if perchlorate was present in the mobile phase (buffer system 1). In contrast, the separation obtained between 22k hGH and met-hGH at neutral pH (Fig. 1d) was lost if perchlorate was added to buffer system 2 (data not shown).

The recoveries of bhGH using various stationary and mobile phases are given in Table I. For the silica columns, no relationship was found between pore size and

TABLE I

RECOVERY OF bhGH USING VARIOUS STATIONARY AND MOBILE PHASES

Column	*Buffer system*	*Recovery (%)*
Nucleosil C_{18}, 120 Å	1	70
Nucleosil C_{18}, 300 Å	1	90
Nucleosil C_{18}, 500 Å	1	82
Nucleosil C_{18}, 1000 Å	1	75
Nucleosil C_4, 1000 Å	1	77
PLRP-S, 100 Å	4	37
PLRP-S, 300 Å	4	37
TSK Phenyl 5PW RP	3	68
TSK Phenyl 5PW RP	4	63

TABLE II

EFFECT OF COLUMN DWELL-TIME ON RECOVERY

After sample application the flow was stopped for 1, 2, 4 or 16 h before gradient elution. The area under the UV curve was measured and compared with that obtained when the column was eluted immediately after sample application. Column: TSK Phenyl 5 PW RP. Mobile phase: (A) $(NH_4)_2HPO_4$–H_3PO_4 (pH 7.0) (B) 90% aqueous acetonitrile with a gradient from 25 to 50% B. Room temperature. Sample: 20 μg of bhGH.

Column dwell-time (h)	*UV peak area (% of dwell time = 0 h)*
1	100
2	100
4	94
16	90

recovery, the optimum recovery (and resolution) being obtained using the 300 Å column. For the resin-based columns, similar recoveries were obtained with the 100 Å and the 300 Å PLRP-S columns. All hGH separations were bettter when using the TSK Phenyl 5PW RP column, which also could be eluted with a higher recovery.

hGH seems to be stable to the chromatographic conditions used, *i.e.*, binding and elution with acetonitrile. If the hGH sample was loaded at the top of the TSK Phenyl 5PW RP column and the elution was stopped for 1, 2, 4 or 16 h before the gradient elution was applied, no difference in peak area was obtained (compared with the area obtained after immediate elution) when the column dwell time was 2 h (Table II).

In order to improve the separation, a number of mobile phase additives were evaluated for both the silica-based and resin based stationary phases: urea, dimethyl- and tetramethylurea, ethylene glycol, diethylene glycol, trifluoroacetic acid, formic acid, acetic acid, citric acid, phosphoserine, tributylammonium phosphate and several

TABLE III

COMPARISON BETWEEN hGH ANALYSES WITH RESPECT TO SEPARATION, DETECTION LIMIT AND TOTAL SAMPLE CAPACITY

Technique	*Separation principle*	*Number of components detected in phGH*	*Load (μg)*	*Detection limit of minor components (%)*	*Quantitative*	*Sample capacity/ 24 h*
GPC	Size	4	20	0.2	+	24
HPIEC	Charge	5	500	0.1	+	48
HIC	Hydrophobicity	2	20	1	+	24
IEF	p*I*	10–20	3	0.1	–	180
DISC	Charge/size	3	500	1	–	32
Chromatofocusing	p*I*	3	200	0.2	+	12
RP-HPLC	Hydrophobicity	10–12	20	0.1	+	24

* He = Heterogeneous; Ho = homogeneous.

** (+) = Partial separation.

alkylsulphonates (C_4–C_{12}) were added to buffer systems 1 and 3, but the resulting phGH and 22k hGH–met-hGH separations were either identical or poorer.

The optimized RP-HPLC analyses shown in Figs. 1 and 2 were compared with other commonly used hGH analyses [gel permeation chromatography (GPC), high-performance ion-exchange chromatography (HPIEC), hydrophobic interaction chromatography (HIC), isoelectric focusing (IEF), disc electrophoresis and chromatofocusing] with respect to separation capacity, speed and total sample capacity (see Table III).

Together with IEF, RP-HPLC yields the most detailed characterization of phGH, but only RP-HPLC will separate met-hGH and 22k hGH. Re-chromatography of the 22k hGH main fraction using any of the described RP-HPLC systems resulted in a single component, whereas a number of minor fractions were observed if a 22k hGH fraction isolated from IEF was re-focused. The reasons for such "ghost bands" may be degradation of the hGH molecule during the focusing procedure and/or reactions between the ampholytes and the sample molecules.

In HPIEC analyses of phGH, hGH-related substances eluting at higher ion strengths than 22k hGH are normally found. These hGH derivatives will focus at a more acidic p*I* in IEF, and are normally considered to be deamidated forms of 22k hGH. None of the RP-HPLC analyses described above were able to separate 22k hGH and deamidated 22k hGH.

DISCUSSION

Although the separation capacity of the RP-HPLC methods described here seems to be superior to those of other published RP-HPLC analyses of hGH, it has not been possible to obtain a similar separation level as that reported for RP-HPLC analyses of polypeptides with considerably lower molecular weights. For insulin, (51 amino acids, MW 6 kD), several RP analyses have demonstrated that insulin peptide easily can be separated from monodesamido-insulin and extended insulins (*i.e.*, mono- and diarginine-insulin)[16].

Separation of met/22k	*Separation of 22k/ desamido 22k*	*Separation of 20k/22k*	*RP-HPLC analyses of major peak**	*Recycling of major peak**	*Method*
–	–	(+)**	He	Ho	TSK 2000 SW, phosphate (pH 7.2)
–	+	+	He	Ho	Mono Q/acetate
–	–	–	He	?	TSK Phenyl 5 PW, 1.8 *M* $(NH_4)_2SO_4 \rightarrow 0$ *M*
–	+	+	Ho	He	pH 4–6/7 *M* urea
–	+	(+)	He	?	pH 8.3
–	+	(+)	He	?	Mono P/pH 4–6/7 *M* urea
+	–	+	Ho	Ho	See systems 1–4

As it was possible to separate hGH (191 amino acids, MW 22 kD) from met-hGH (hGH extended with a single methionine), it is surprizing, that no separation could be obtained between hGH and deamidated hGH. The reason for this missing separation could be that the deamidation site(s) are buried in the molecule and therefore not readily exposed to the stationary phase. Although acetonitrile is generally assumed to attack the tertiary structure of proteins, hGH probably withstands the chromatographic conditions without a substantial loss of structure, as indicated in the column dwell-time experiments (Table I).

Quantitation of most hGH-related substances can be obtained after RP-HPLC, but in order to detect the amount of deamidated hGH, HPIEC must be applied. However, as UV detection at 200–215 nm cannot be used in the published HPIEC analyses, the method is less sensitive, and fewer hGH-related substances are detected than with RP-HPLC

In order to detect all components present in a given hGH sample, more than one separation principle must therefore be applied.

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