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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF AMINO ACIDS, PEPTIDES AND PROTEINS

LXIII*. REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC CHARACTERISATION OF SEVERAL POLYPEPTIDE AND PROTEIN HORMONES

BORIS GREGO** and MILTON T.W. HEARN*

St. Vincent's School of Medical Research, St. Vincent's Hospital, Victoria Parade, Fitzroy 3065 (Australia)

SUMMARY

The chromatographic behaviour on alkylsilicas of a variety of hormonal proteins is described. Optimization of resolution and recovery of these protein hormones, which included porcine relaxins, human chorionic gonadotropin, human placental lactogen, pituitary derived growth hormone and adenohypophyseal glycoprotein hormones, was achieved by manipulation of both mobile and stationary phase parameters. With standard stainless-steel analytical columns (10—30 cm × 0.4 cm) packed with meso- or macro-porous *n*-alkylsilica supports these proteins can be readily fractionated at the semi-preparative level with separation times generally under 90 min using elution systems directly compatible with subsequent methods of primary structure determination or biological functional analysis. The effects of changes in several experimental parameters on peak symmetry, retention and recovery are described.

INTRODUCTION

At the present time reversed-phase high-performance liquid chromatography (RP-HPLC) is the most widely used liquid chromatographic technique for the separation at the micro- and semi-preparative level of peptides and small globular proteins [1]. Selectivity and retention of peptides and proteins on microparticulate chemically bonded alkylsilicas can be manipulated by a variety of mobile phase parameters including the chemical nature and con-

*For Part LXII, see ref. 49.
**Present address: Ludwig Institute for Cancer Research, Melbourne Tumour Biology Unit, Post Office Royal Melbourne Hospital, Victoria 3050, Australia.

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centration of added pairing ion or buffer species, the pH, and the nature and concentration of the organic solvent modifier [1—9]. Under neat aqueous conditions peptides, polypeptides and proteins in general show considerable retention to alkylsilicas. Because of complex multi-site binding phenomena associated with the solute—stationary phase interaction, isocratic elution conditions with aquo—organic solvent combinations rarely permit adequate resolution and recovery of peptide or protein mixtures. However, gradients in organic solvent modifiers, pH and buffer ions allow rapid separation on bonded *n*-alkylsilicas of peptides and proteins encompassing a wide range of differences in hydrophobicities, molecular weight and subunit structure. In associated studies [4—9], we have reported rigorous procedures for the optimization of peptide resolution on porous microparticulate alkylsilicas. In this investigation these procedures have been applied to the RP-HPLC fractionation of a variety of hormonal peptides and proteins with the recovered fractions obtained in a form suitable for structural and biological characterization.

EXPERIMENTAL

Chemicals and reagents

Water was distilled and deionized using a Milli-Q system (Millipore, Bedford, MA, U.S.A.). Acetonitrile was HPLC grade obtained from Waters Assoc. (Milford, MA, U.S.A.) or Burdick and Jackson Labs., (Muskegon, MI, U.S.A.). Orthophosphoric acid, acetic acid, trifluoroacetic acid, ammonium bicarbonate and ammonium acetate were AnalaR grade from BDH (Poole, U.K.) or May and Baker (Dagenham, U.K.). Triethylamine, trifluoroethanol and *tert.*-butanol were from Sigma (St. Louis, MO, U.S.A.). Partially purified porcine relaxins, which had been fractionated by the Sherwood and O'Byrne method [10], were a generous gift from Dr. R. Bradshaw, University of California at Davis, CA, U.S.A. Human chorionic gonadotropin (hCG), human placental lactogen (hPL), human growth hormone (hGH), human prolactin (hPrL), human follicle stimulating hormone (hFSH), human thyroid stimulating hormone (hTSH) and human luteinizing hormone (hLH) and their radio-iodinated analogues were prepared in this laboratory by established procedures. The human growth hormone 20K variant was isolated according to the method of Chapman et al. [11].

Apparatus

All chromatographic data were obtained on a Waters gradient elution system which consisted of two Model M6000A solvent delivery pumps, a M660 solvent programmer, a U6K universal sample injector, a Model M450 variable-wavelength UV monitor and a M720 data module. Sample injections were made with SGE syringes (Melbourne, Australia). The pH measurements were performed with a Radiometer PHM-64 meter equipped with a combination glass electrode. The μBondapak C_{18} and alkylphenyl columns were purchased from Waters Assoc. The in-house columns were made by bonding trimethylchlorosilane, *n*-butyldimethylchlorosilane and *n*-octyldimethylchlorosilane onto LiChrospher silica (Merck, Darmstadt, F.R.G.) of two nominal pore diameters,

10 nm and 50 nm and of nominal particle size 10 μm. The ligand density of the *n*-butyl- and *n*-octylsilica phases were 3.3, 3.8, 3.4 and 3.6 μmol/m^2, respectively. Radioactivity was determined on a Packard gamma spectrometer.

Methods

Bulk solvents and appropriate mobile phases were prepared and degassed as reported previously [5—8]. Columns were equilibrated for at least 30 min at 2 ml/min between gradient elution experiments and for at least 60 min for the isocratic elution experiments as well as for all systems containing *tert.*-butanol, trifluoroethanol and triethylamine. All chromatograms were prepared at 18°C. Amino acid analysis was performed with a Durrum D500 amino acid analyser, using standard hydrolysis conditions [12].

RESULTS AND DISCUSSION

RP-HPLC of porcine relaxins

Although relaxin was first described nearly sixty years ago by Hisaw and co-workers [13, 14], its role in human physiology remains to be completely elucidated. During gestation, relaxin levels increase in the ovaries and blood and produce relaxation of the symphysis, inhibition of uterine contractility, and softening of the cervix. Relaxin was first isolated from sow corpora lutea and later from other species [15—17] by a combination of conventional gel permeation and ion-exchange chromatographic procedures. Several reports have demonstrated [18—20] that the relaxin activity obtained by these conventional chromatographic procedures is shared by several low molecular weight basic proteins. The porcine relaxin preparations used in the present study were isolated by CM-cellulose chromatography based on the procedure of Sherwood and O'Byrne [10]. These preparations were believed to be microheterogeneous on the basis of several criteria including charge electrophoresis, isoelectric focusing and carboxypeptidase cleavage. The microheterogeneity was presumed to arise as a consequence of post-translational processing which leads to partial deletion of C-terminal argininyl peptides from the relaxin B-chain. The resolution on alkylsilicas under isocratic or gradient aquo—organic solvent conditions of such closely related polypeptides, differing mainly in arginine content, is known [1, 2] to be very responsive to pH and ionic additive effects. Selectivity advantages can be taken from these effects by employing ternary gradient elution systems derived, for example, from combinations of ammonium acetate, trifluoroacetic acid and an organic solvent modifier. A further advantage of such volatile systems is the relative ease by which the separated polypeptides can be recovered from the chromatographic fractions by lyophilisation. Based on preliminary experiments designed to examine band width, relative retention and recovery, a linear 0—50% acetonitrile gradient from 100 m*M* ammonium acetate, pH 7.0, to 15 m*M* trifluoroacetic acid, pH 2.0, was selected as a suitable elution system for the porcine relaxin preparations. The gradient slope and flow-rate were then chosen on the basis of relative resolution, i.e. from the peak capacity dependence on gradient *b*-values [7—9]. Fig. 1 shows the chromatographic profiles of three different relaxin preparations, obtained from the CM-cellulose ion-exchange stage,

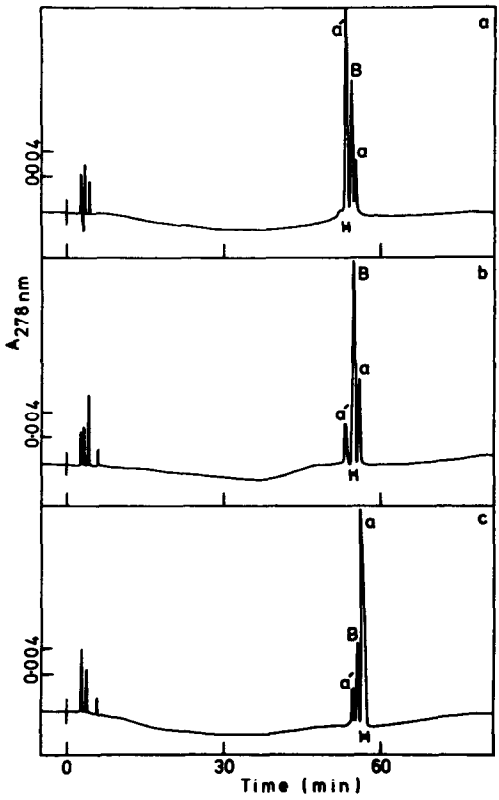

Fig. 1. Separation of porcine relaxin species by RP-HPLC. Chromatographic conditions: column, μBondapak alkylphenyl (30 × 0.4 cm, d_p 10 μm); flow-rate, 1.2 ml/min; linear 90-min gradient from 0 to 100% B, where solvent A was 100 m*M* ammonium acetate, pH 7.0, and solvent B was 50% acetonitrile—50% water—15 m*M* trifluoroacetic acid, pH 2.0; detection 278 nm at 0.04 a.u.f.s. Sample loadings: (a) CM-cellulose fractionated porcine relaxin a', 110 μg in 100 μl; (b) CM-cellulose fractionated porcine relaxin B, 125 μg in 100 μl; (c) CM-cellulose fractionated procine relaxin a, 115 μg in 100 μl. The bars under the peaks indicate fractions collected for amino acid analysis, and their compositions are given in Table I.

separated on a μBondapak alkylphenyl support under the above gradient elution conditions. Fractions corresponding to each profile were collected and subjected to amino acid analysis (Table I). From comparisons of the elution profiles and the composition analysis it was apparent that each of the CM-cellulose preparations contained three porcine relaxin species. When the results of the composition analyses of these three porcine relaxin species were compared with other published data it was evident that the material called relaxin "B" in this study corresponded to the porcine relaxin species sequenced by Schwabe et al. [21—23], the species labelled "B" by Frieden et al. [24], and the species designated "CM-B" by Sherwood and O'Byrne [10]. A feature of all of these porcine relaxin species, besides the other amino acids they share in common, is the presence of five arginine residues. The composition of the species designated "relaxin a" in Fig. 1 and Table I corresponded to the porcine relaxin preparation sequenced by James et

TABLE I

AMINO ACID COMPOSITIONS OF PORCINE RELAXIN SPECIES

Amino acid compositions of relaxin peptides from the chromatographic profiles shown in Fig. 1. Compositions are given for the main peaks only. The minor peaks were contaminants of either relaxins a, a′ or B. The data are presented as nmol of amino acid residue per nmol of lysine where the number of lysines per molecule was set equal to three. Total protein recovered in the collected fractions was: relaxin a′, 95 μg or 86%; relaxin B, 112 μg or 90%; relaxin a, 105 μg or 91%. nd = not determined.

Amino acid	Present study			Ref. 24			Ref. 10		Refs. 21—23	Ref. 25
	a′	B	a	A	B	C	CM-a	CM-b		
D	2.6	3.3	2.7	2.9	2.9	3.0	2.8	2.9	3	3
T	1.6	2.7	2.1	2.1	2.1	1.7	2.3	1.9	2	2
S	2.7	4.0	4.0	3.2	3.4	2.8	2.8	2.8	3	4
E	4.1	5.2	5.2	4.8	4.8	4.7	4.7	4.7	5	5
P	0	0	0	1.2	0	1.0	0	0	0	
G	2.7	4.6	4.9	3.8	3.6	3.4	3.6	3.2	3	4
A	1.8	3.0	2.7	2.5	2.6	3.1	2.7	2.3	2	2
C	nd	nd	nd	5.3	6.0	5.4	4.8	4.9	6	6
V	2.7	3.6	2.7	3.7	3.6	3.2	3.7	3.5	4	4
M	0.8	0.9	1.0	0.9	0.8	0.8	0.8	0.8	1	1
I	3.0	3.2	2.8	3.6	3.4	2.8	3.3	3.2	4	4
L	3.1	3.6	3.8	4.1	4.1	3.9	3.9	3.8	4	4
Y	0	0	0	0	0	0.2	0	0	0	0
F	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1	1
H	0	0	0	0	0	0.4	0	0	0	0
K	3.0	3.0	3.0	3.3	3.1	3.3	3.1	3.2	3	3
W	nd	nd	nd	1.6	1.4	1.0	nd	nd	2	2
R	3.9	5.3	6.0	5.6	5.0	3.9	5.3	4.5	5	6

al. [25], and that labelled "A" by Frieden et al. [24]. However, our relaxin "a" preparation lacked a proline residue as reported by Frieden et al. [24]. This relaxin "a" species contains six arginine residues. Finally, the composition of the species designated relaxin "a′" in Fig. 1 and Tabel I corresponded to the material labelled "C" by Frieden et al. [24], except again our preparation lacked the proline residue as reported by these workers. This relaxin "a′" species contains four arginine residues. It is obvious from our results and other published data (Table I) that there are a number of different but chemically related relaxin polypeptides which are only partially resolved by CM-cellulose fractionation, but which can be well separated under the above RP-HPLC conditions. Whether these three porcine relaxin species arise as breakdown products during the isolation procedure or are true biosynthetic products remains, however, to be clarified.

The conditions used to generate the chromatograms in Fig. 1 gave excellent recoveries as determined by quantitive amino acid analysis, for example, recoveries of 86% for relaxin "a′", 90% for relaxin "B", and 91% for relaxin "a", were obtained. As part of the preliminary experiments other chromatographic conditions were also examined, including gradients in acetonitrile with low pH mobile phases consisting of either 0.1% orthophosphoric acid or 0.1%

trifluoroacetic acid. Under these acidic conditions it was not possible to resolve the three porcine relaxin species on several different stationary supports (e.g. μBondapak C_{18}, μBondapak alkylphenyl, and *n*-octyl LiChrospher 100). Similar lack of discrimination of microheterogeneous relaxin forms has been observed by Reinig et al. [15], who used 10% acetic acid as the mobile phase buffer in the isolation of shark relaxin and its two subunit components on a μBondapak C_{18} support. In other experiments, we examined the efficacy of 200 m*M* ammonium bicarbonate at pH 7.8 as the primary mobile phase buffer, but resolution of the "a" and the "a′" relaxin species was incomplete (Fig. 2) even when shallow gradients in acetonitrile concentration (e.g. 0.12%/ml/min) were used, the resolution was inferior to the results shown in Fig. 1 with either μBondapak C_{18} or *n*-octyl LiChrospher 100 support material. Since the porcine relaxins are basic solutes it was anticipated that chromatographic selectivity could be enhanced further by the use of an anionic pairing ion such as heptanesulphonate at a concentration up to 15 mmol/dm^3 in the mobile phase. Previous studies [5, 12, 26, 27] have demonstrated that increased column selectivity generally occurs with unprotected peptides when low concentrations of anionic pairing ions are used with acidic (ca. pH 2—3) mobile phase conditions. However, the three porcine relaxin species coeluted (data not shown) from the three different stationary phases examined when heptanesulphonate concentrations of 10 mmol/dm^3 were employed with aquo—acetonitrile gradient conditions otherwise identical to those used for the separations shown in Fig. 1.

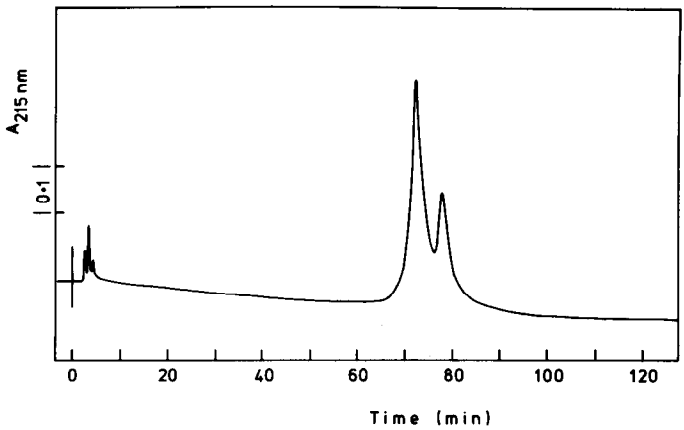

Fig. 2. Separation of CM-cellulose fractionated porcine relaxin a and porcine relaxin a′ by RP-HPLC with an ammonium bicarbonate buffer in the mobile phase. Chromatographic conditions: column, C_8 alkyl chain bonded to 10 nm pore size LiChrospher silica, (15 × 0.4 cm, d_p 10 μm); flow-rate, 1.0 ml/min; linear 150-min gradient from 45 to 80% B, where solvent A was 200 m*M* ammonium bicarbonate—water, pH 7.8, and solvent B was 50% acetonitrile—50% water—200 m*M* ammonium bicarbonate; detection, 215 nm at 1.0 a.u.f.s.; sample loading, 125 μg of a mixture of porcine relaxins a and a′ in a volume of 100 μl.

RP-HPLC of human placental polypeptide hormones

In pregnancy there are remarkable alterations in hormone production. The human placenta, for example, secretes large quantities of a variety of both steroid and polypeptide hormones, including chorionic gonadotropin (hCG)

and human placental lactogen (hPL). The glycoprotein hCG, in common with the pituitary glycoprotein hormones, possesses a quaternary structure characterized by two dissimilar polypeptide chains, designated α- and β-subunits. The α-subunit consists of 92 amino acids and is very similar in sequence to the α-subunits of the pituitary glycoprotein hormones [28, 29], while the β-chain consists of 145 amino acids [28, 30, 31] and shows some sequence homology with other glycoprotein hormone β-subunits [31]. In the human hCG has luteotropic activity and is believed to stimulate the foetal gonads to secrete steroids at a time prior to the secretion of LH by the foetal pituitary [32].

Fig. 3 shows the chromatographic profiles of preparations of hCG and its α- and β-subunits under mobile phase conditions identical to those used above for the separation of the porcine relaxin species. Although the α- and β-subunit preparations used in this investigation were believed by other criteria to be homogeneous [28, 29] each preparation gave two chromatographic peaks on

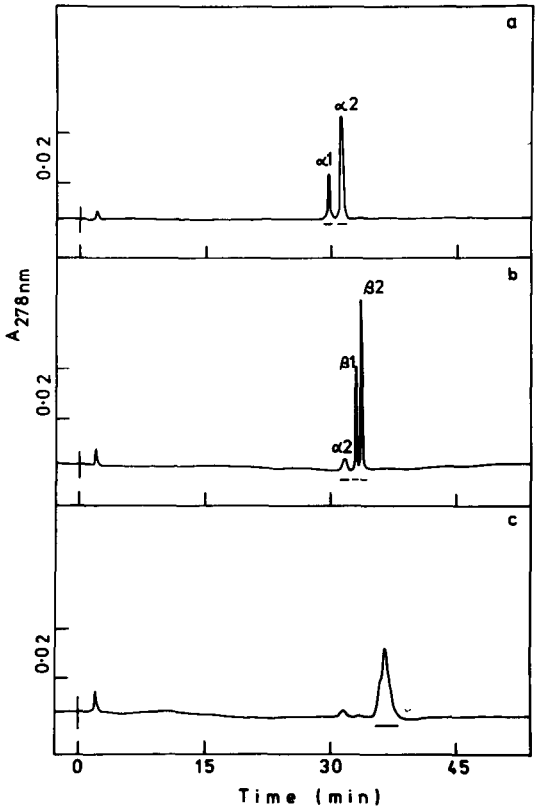

Fig. 3. RP-HPLC of preparations of human chorionic gonadotropin (hCG) and its α- and β-subunits. Chromatographic conditions: column, μBondapak alkylphenyl (30 × 0.4 cm, d_p 10 μm); flow-rate, 1.2 ml/min; linear 90-min gradient from 0 to 100% B, where solvent A was 100 m*M* ammonium acetate—water, pH 7.0, and solvent B was 60% acetonitrile—40% water—15 m*M* trifluoroacetic acid, pH 2.0; detection, 278 nm at 0.04 a.u.f.s. Samples: (a) 150 μg of hCG α-subunit in 100 μl; (b) 150 μg of hCG β-subunit in 100 μl; (c) 250 μg of hCG in 150 μl. Peaks underlined with bars were collected and analyzed for amino acid composition, and the data are summarized in Table II.

TABLE II

AMINO ACID COMPOSITIONS OF PLACENTAL HORMONES

Amino acid analysis of hCG, hCG subunits and hPL after chromatography on reversed-phase, microparticulate silica supports. Peaks were collected from the bar regions of the profiles in Figs. 3 and 4. For hCG the results are presented as nmol of amino acid residue per nmol of alanine where the integer value of alanine was set to 5 in the α-subunit, 8 in the β-subunit, and 13 for the intact hCG. Published composition values are from the amino acid sequence data given in ref. 29. Total recovery of protein was 126 μg or 83% for the hCG α-subunit preparation, 134 μg or 89% for the hCG β-subunit preparation, and 221 μg or 88% for the parent hCG molecule. For hPL the results are presented as nmol of amino acid residue per nmol of alanine where the integer value of alanine was set at 6. Published composition values for hPL are from refs. 36 and 37. Total recovery of protein was 159 μg or 91% of the starting material. nd = not determined.

Amino acid	hCG α-subunit			hCG β-subunit			hCG		hPL	
	1	2	predicted	1	2	predicted	observed	predicted	observed	predicted
D	6.4	6.3	6	12.4	11.1	11	17.3	17	20.2	23
T	8.1	8.3	8	9.7	9.5	10	17.9	18	11.9	13
S	7.9	8.0	8	11.5	10.9	13	19.9	21	16.9	18
E	9.8	10.1	9	9.7	9.1	9	19.2	18	20.1	24
P	9.4	9.1	7	23.1	25.1	22	31.1	29	6.9	5
G	5.0	5.0	4	9.3	8.6	8	14.1	12	6.9	7
A	5.0	5.0	5	8.0	8.0	8	13.0	13	nd	4
C	nd	nd	10	nd	nd	12	nd	22	nd	4
V	7.2	7.1	7	11.5	12.0	12	19.2	19	6.9	7
M	2.8	2.9	3	1.5	1.8	1	5.9	4	7.2	6
I	2.7	1.2	1	5.3	6.7	5	7.2	6	6.9	6
L	5.9	4.5	4	11.3	13.8	12	17.2	16	27.6	25
Y	5.7	3.6	4	5.0	5.0	3	8.6	7	9.1	8
F	6.0	4.0	4	1.9	2.1	2	8.1	6	13.3	11
H	2.7	3.1	3	2.0	1.1	1	4.3	4	6.6	7
K	6.0	6.2	6	4.6	4.0	4	9.9	10	9.1	9
W	nd	nd	0	nd	nd	0	nd	0	nd	1
R	3.2	3.3	3	11.5	11.5	12	15.3	15	11.2	10

RP-HPLC separation. Composition analysis of the collected fractions (Table II) showed that the two peaks corresponding to the α-subunit preparation had very similar amino acid content. Similarly, the two peaks generated from the β-subunit preparation also correlated closely in composition. These double peaks from each apparently homogeneous subunit may be due to either partially deamidation of the subunits during the isolation procedure, or alternatively to differences in the glycosylation state. As is evident from Fig. 3, these RP-HPLC procedures enable complete resolution of the two α-subunit isoforms from the two β-subunit isoforms and also from the parent hCG protein. Recoveries were 83%, 89% and 88% for the α-subunit, β-subunit and parent hCG, respectively. Even with small-pore alkylsilicas such as the μBondapak alkylphenyl support excellent peak shape can be achieved with the 100 m*M* ammonium acetate, pH 7.0, to 60% acetonitrile—15 m*M* trifluoroacetic acid, pH 2.0, elution condition where gradients in both composition and pH are achieved. These results can be contrasted with the observations of

Putterman et al. [33] who have reported a similar elution order pattern for the subunits (i.e. $t_g\alpha < t_g\beta$) but significantly lower resolution due to greater band broadening in the separation of hCG and its subunits by RP-HPLC procedures using a gradient in acetonitrile containing 0.1% trifluoroacetic acid alone as the mobile phase buffer, and a μBondapak C_{18} support.

Human placental lactogen (hPL) is a single-chain polypeptide hormone of 191 amino acids devoid of carbohydrate moieties [34]. There is considerable sequence homology between hPL and human growth hormone (HGH) with 162 residues of the primary sequence being identical in the two proteins [35—37]. Fig. 4 shows a further example of the utility of the ammonium acetate—trifluoroacetic acid system, namely, the chromatography of hPL, previously purified by established methods [36], on a μBondapak alkylphenyl support using the standard 0—50% acetonitrile gradient. Composition analysis of the collected peak fraction is given in Table II. The mass recovery of hPL was 91% of the protein loaded.

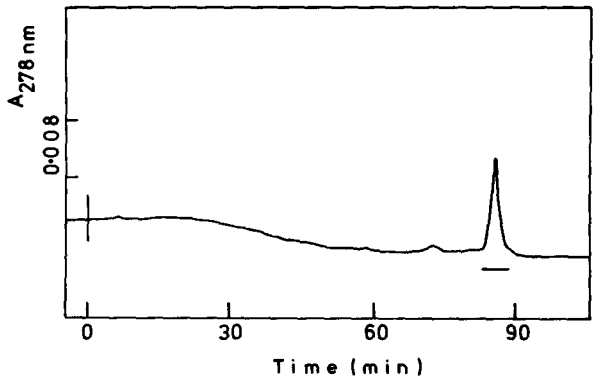

Fig. 4. RP-HPLC of human placental lactogen (hPL) preparation. Chromatographic conditions: column, μBondapak alkylphenyl (30 × 0.4 cm, d_p 10 μm); flow-rate, 1.2 ml/min; linear 90-min gradient from 0 to 100% B, where solvent A was 100 m*M* ammonium acetate—water, pH 7.0, and solvent B was 60% acetonitrile—40% water—15 m*M* trifluoroacetic acid, pH 2.0; detection, 278 nm at 0.04 a.u.f.s. Sample, 175 μg of hPL in 100 μl. The peak underlined was collected and analyzed for amino acid composition, and the data are given in Table II.

RP-HPLC of human pituitary polypeptide hormones

The human adenohypophysis contains several polypeptide hormones of established functional significance, including the two-subunit glycoproteins follicle stimulating hormone (hFSH), thyroid stimulating hormone (hTSH) and luteinising hormone (hLH), and the single-chain non-glycosylated proteins, growth hormone (hGH) and prolactin (hPrL). Our aim was to devise optimal chromatographic conditions for the recoveries of these protein hormones, compatible with subsequent structural or biological analysis. A stringent test of any chromatographic system is the requirement of high recovery of radiolabelled solutes such as radio-iodinated polypeptides which on a mass basis may be present in the sample to be loaded and the eluted fractions in only trace quantities.

Table III summarizes the recoveries of several different ^{125}I-labelled poly-

TABLE III

RECOVERIES OF RADIO-IODINATED POLYPEPTIDE HORMONES UNDER DIFFERENT ELUTION CONDITIONS

Key to the chromatographic conditions: Condition 1. μBondapak C_{18}, flow-rate 2.0 ml/min, linear 60-min gradient from 0 to 100% B, where solvent A was 15 m*M* orthophosphoric acid—water, pH 2.25, and solvent B was 50% acetonitrile—50% water—15 m*M* orthophosphoric acid. Condition 2. μBondapak C_{18}, flow-rate 2.0 ml/min, linear 60-min gradient from 0 to 100% B, where solvent A was 200 m*M* ammonium bicarbonate—water, pH 7.8, and solvent B was 50% acetonitrile—50% water—200 m*M* ammonium bicarbonate. Condition 3. μBondapak alkylphenyl, flow-rate 2.0 ml/min, linear 60-min gradient from 0 to 100% B, where solvent A was 200 m*M* ammonium acetate—water, pH 7.0, and solvent B was 60% acetonitrile—40% water—80 m*M* acetic acid, pH 3.5. Condition 4. C_1 alkyl chain bonded to 50 nm pore size LiChrospher silica, flow-rate 2.0 ml/min, linear 60-min gradient from 0 to 100% B, where solvent A was 200 m*M* ammonium bicarbonate—water, pH 7.8, and solvent B was 50% acetonitrile—50% water—200 m*M* ammonium bicarbonate. All of the μBondapak columns were of the same dimensions, 30 × 0.4 cm with d_p 10 μm, while the C_1 bonded phase was 7.5 × 0.4 cm, d_p 10 μm. In all experiments 1-min fractions were collected and counted in a γ-counter.

Chromatographic condition	^{125}I-labelled hormone	Recovery (%)
1	hGH	10
	hPrL	50
2	hGH	75
	hPrL	61
	hTSH	82
	hFSH	85
	hLH	65
3	hGH	60
	hPrL	97
	hTSH	94
	hFSH	99
	hLH	95
4	hTSH	85
	hLH	98

peptide hormones under a variety of chromatographic conditions. One of the constraints which must be applied to the reversed-phase chromatography of glycoprotein hormones such as FSH, TSH and LH, concerns the choice of the mobile phase pH. At inappropriate mobile phase pH values dissociation of the native glycoprotein hormones to their respective subunits may occur. In comparison the single-chain hormones such as hGH and hPrL are relatively stable for short periods in mildly acidic conditions, such as 0.1% phosphoric or 0.1% trifluoroacetic acid, but over some acidic pH ranges these proteins exhibit low solubility or may partially deamidate [12]. Load-dependent recovery effects may be a further complication in ultramicropreparative RP-HPLC separations of trace quantities of these radiolabelled polypeptide hormones. The results obtained with radio-iodinated hGH and hPrL with a μBondapak C_{18} column and a gradient of acetonitrile containing 0.1% orthophosphoric acid as

the mobile phase ionic additive (elution condition 1 in Table III) are typical examples. Thus, with sample loadings of [^{125}I]hGH or [^{125}I]hPrL equivalent to ca. 5 pg of protein the recovery of [^{125}I]hGH was 10% whilst the recovery of hPrL was 50%. The same chromatographic conditions have previously given good recoveries (as assessed by quantitative amino acid analysis) of hGH (ca. 90%) when the sample loaded was in the range 5 μg to 3 mg [38]. Other groups [39, 40] have also reported similar observations regarding load-related recoveries of certain proteins chromatographed on reversed-phase alkylsilicas.

Volatile mobile phases are commonly used for preparative separations because of their ease of removal. In previous studies [12, 38, 41] we have extensively used ammonium bicarbonate as a mobile phase buffer in the RP-HPLC of peptides and proteins. Using the same μBondapak C_{18} column as employed with the 0.1% orthophosphoric acid elution system, the recoveries of radio-iodinated HGH and hPrL improved significantly up to 75% and 61% respectively with 200 m*M* ammonium bicarbonate as the mobile phase buffer with a 0—50% acetonitrile gradient (chromatographic condition 2 in Table III). The recoveries of the radio-iodinated glycoprotein hormones hTSH, hFSH and hLH ranged from 65% to 85% under these chromatographic conditions. Fig. 5

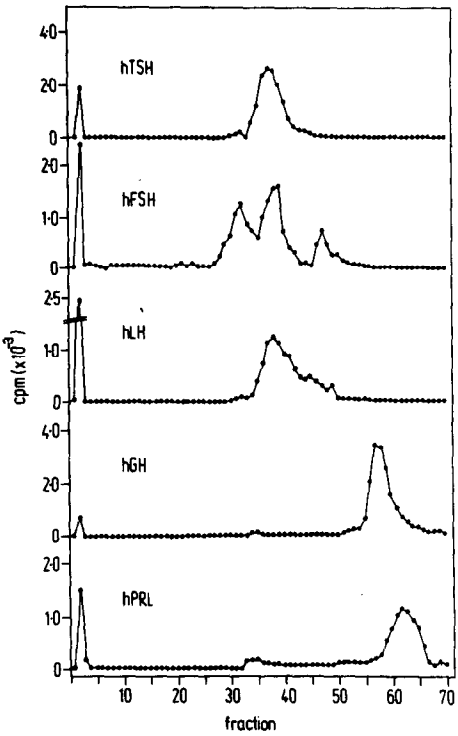

Fig. 5. RP-HPLC of iodinated pituitary polypeptide hormones under constant pH elution conditions. Chromatographic conditions: column, μBondapak C_{18} (30 × 0.4 cm, d_p 10 μm); flow-rate, 2.0 ml/min; linear 60-min gradient from 0 to 100% B, where solvent A was 200 m*M* ammonium bicarbonate—water, pH 7.8, and solvent B was 50% acetonitrile—50% water—200 m*M* ammonium bicarbonate. Fractions were collected at 1-min intervals and counted directly in a γ-spectrometer. Recoveries of labelled hormones are given in Table III.

shows the elution profiles obtained under these chromatographic conditions for these five polypeptide hormones. As discussed elsewhere [42] the peak broadening observed for these radio-iodinated hormones appears to be a consequence of several factors. First, iodination of proteins which contain several accessible tyrosine residues leads to the generation of multiple iodinated species which may be only partially resolvable under a particular chromatographic condition. Second, pituitary glycoprotein hormones in particular exhibit considerable microheterogeneity in terms of their charge and glycosylation patterns [43]. Third, peak shapes for globular proteins above 20 Kdaltons are very responsive to mobile phase mediated phenomena which affect the diffusion coefficients of these solute as well as the kinetic resistance to migration of the solutes onto and off the alkylsilica support. We have discussed the contribution of resistance to mass transfer between the mobile and stationary phases for protein solutes in a previous report [44]. Under otherwise fixed eluent conditions for solutes with small diffusion coefficients such as proteins, peak shape may be improved by reducing both particle diameter of the support and flow-rate, as well as by improving the mass transfer properties of the stationary phase. The latter requirement may be only partly achieved by increasing the pore size of the support [44]. Much more significant control over band broadening due to stationary phase effects can be achieved when less heterogeneously bonded or dynamically coated non-polar surfaces are employed thereby reducing the extent of multiple-site interaction with complex protein solutes [44, 45]. The participation of all of these factors can give rise to apparent peak broadening in the gradient elution RP-HPLC of these radio-iodinated protein hormones. It was thus of interest to examine the chromatographic behaviour of individual fractions on rechromatography. In all cases examined rechromatography under identical conditions of single fractions from the experiments shown in Fig. 5, resulted in the radio-iodinated component(s) eluting with unchanged retention, i.e. at the same fraction number if the flow-rate and fraction size was constant, but with significantly improved peak shape in many cases equivalent to that observed for the unlabelled parent protein. There are two precautions regarding the use of high molarity ammonium bicarbonate as a mobile phase buffer for protein separations by RP-HPLC which should be mentioned. First, its solution pH of 7.8 is close to or above the upper pH limit of some commercial silica-based supports. Second, it is our experience that highly basic proteins may exhibit very low recoveries with this buffer composition and pH due to titration of cationic side chains on the proteins resulting in a less polar species [5, 26, 27] and titration of weak acidic groups on the alkylsilica support resulting in non-ideal reversed-phase behaviour. Chromatographic conditions consisting of gradients in acetonitrile concentration and pH similar to those described for the porcine relaxins and hCG were also found effective for the separations of several pituitary polypeptide hormones. For example, with linear gradients of 100 m*M* ammonium acetate, pH 7.0, to 60% acetonitrile in 80 m*M* acetic acid, pH 3.5, recoveries of several radio-iodinated proteins were over 90% except for hGH (Table III) although significant band broadening was still evident (Fig. 6) in all cases.

The above results are in accord with our previous findings [4—9, 12] which

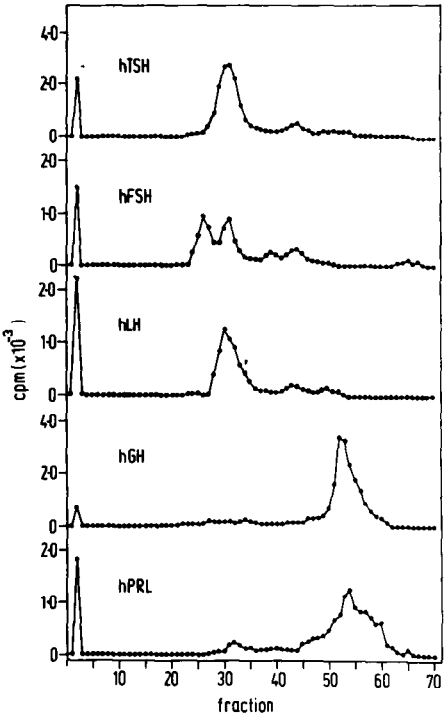

Fig. 6. RP-HPLC of iodinated pituitary polypeptide hormones, under pH and solvent gradient conditions. Chromatographic conditions: column, μBondapak alkylphenyl (30 × 0.4 cm, d_p 10 μm); flow-rate, 2.0 ml/min; linear 60 min gradient from 0 to 100% B, where solvent A was 200 m*M* ammonium acetate—water, pH 7.0, and solvent B was 60% acetonitrile—40% water—80 m*M* acetic acid, pH 3.5. 80 m*M* acetic acid is equivalent to ca. 0.5% solution. Fractions were collected at 1-min intervals and counted in a γ-spectrometer. Recoveries of labelled hormones are given in Table III.

demonstrated that retention of polypeptides and proteins on alkylsilicas is to a very large extent dominated by mobile phase effects and in particular by the choice and concentration of the organic solvent modifier. In common with many other globular proteins, the retention of the pituitary protein hormones to alkylsilicas is very responsive to small changes in the water content of binary or ternary aquo—organic solvent mobile phase combinations. For example both the 22-Kdalton hGH and the 20-Kdalton hGH variant exhibited characteristic bimodal retention dependencies on the concentration of the organic solvent modifier when eluted with water—acetonitrile elution systems from butyl- and octyl-bonded silicas of nominal pore sizes of 10 nm and 50 nm (Fig. 7). As can be seen from Fig. 7, with these two proteins there is clearly a significant pore size effect. Even when total column porosities and column packing densities are taken into account, at a given k' value both these proteins elute from the small-pore *n*-butyl- or *n*-octylsilica at a lower acetonitrile concentration than from the corresponding large-pore *n*-alkylsilica. In this retention behaviour, these hGH polypeptides differ from the glycoprotein hormones or other globular proteins such as lysozyme, trypsin or phosphorylase a [46]. Over the range of regular reversed-phase elution behaviour, the pituitary protein

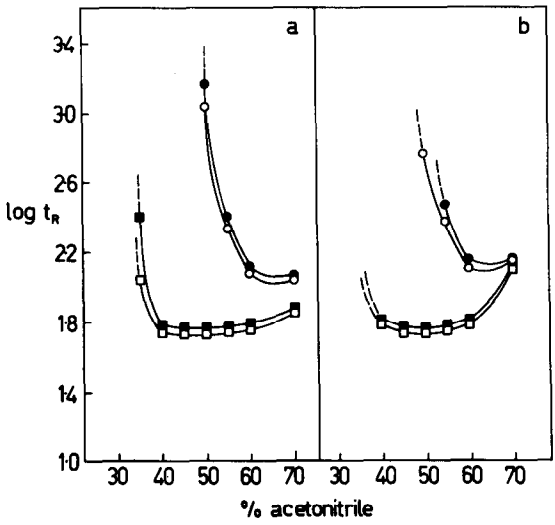

Fig. 7. Dependence of retention of 22K human growth hormone (a) and the 20K human growth hormone variant (b) on the mobile phase acetonitrile concentration. Chromatographic conditions: mobile phase, 50 m*M* NaH_2PO_4—15 m*M* H_3PO_4, pH 2.3, with the mobile phase acetonitrile concentration adjusted between 0 and 70%; flow-rate, 1.0 ml/min; columns, four supports each of a nominal 10-μm particle size but differing in alkyl chain length and particle porosity were used according to the key: □ = C_4 Si-100, ■ = C_8 Si-100, ○ = C_4 Si-500, ● = C_8 Si-500. The same column dimensions (15 × 0.4 cm) were used for each support material.

hormones exhibited curvilinear plots between the logarithmic capacity factor, log k', and the mole fraction of the organic solvent modifier, ϕ, with tangent slopes typical of globular proteins in this molecular weight range, i.e. with S-values between 60 and 80. One consequence of the pronounced retention dependencies of these proteins on the organic solvent content in RP-HPLC systems is that apparent retention times are nearly independent of column length under otherwise fixed gradient elution conditions. For example, under the 200 m*M* ammonium bicarbonate—acetonitrile elution conditions (condition 4 in Table III) the relative retention of the radio-iodinated protein hormones chromatographed on C-1, C-4 and C-18 bonded alkylsilica of 50 nm nominal pore diameter packed into columns of 10 cm length were almost identical to those observed with 30-cm columns of the same internal diameter packed with the same stationary phase.

Finally, the influence of polar modifiers on the resolution and recovery of these proteins was examined. Previously we have shown [47] that polar modifiers such as *tert.*-butanol in low concentrations reduce the retention of small peptides on alkylsilicas presumably by dynamically modifying the stationary phase surface. Addition of *tert.*-butanol or trifluoroethanol at a concentration of 1% (v/v) in the mobile phase (for example condition 2 in Table III) caused a reduction in the recovery of [^{125}I]hGH and [^{125}I]hPrL from 60—70% to about 10—15% of the amount loaded, compared to the corresponding elution condition lacking these polar modifiers as well as reduced resolution. Similarly, addition of 25 m*M* triethylamine at acidic (pH 3.0) and neutral (pH 7.8) conditions did not enhance recoveries as anticipated

on the basis of a decrease in silanol effects although peak shape was improved.

In summary, chromatographic conditions compatible with the RP-HPLC of several polypeptide and protein hormones have been developed. These separation conditions permit rapid high-resolution fractionation of pituitary derived protein hormones and components of placental or ovarian origin. Extension of these studies to large-scale purification of these biologically important proteins will be reported elsewhere [48].

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