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

LXXXV*. SEPARATION OF ISOFORMS OF THE GLYCOPROTEIN HORMONES FROM HUMAN PITUITARY EXTRACTS

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SUMMARY

Procedures for the high-resolution separation of microheterogeneous forms of the glycoprotein hormones, lutropin, follitropin and thyrotropin, present in human pituitary extracts are described. The sequential use of preparative isoelectric focusing and ion-exchange high-performance liquid chromatography (HPLC) allows the further discrimination of the isoforms of these glycoprotein hormones, previously considered homogeneous on the basis of their electrophoretic mobility. The ability of ion-exchange HPLC stationary phases to probe for slight differences in charge asymmetry expressed on the surface of these proteins raises the possibility of their rapid purification via the tandem on-line use of the same ion-exchange support with several different elution systems. Retention maps, illustrating the basis of this approach, are described.

INTRODUCTION

Studies related to the structure–function relationships of the gonadotrophic protein hormones are central to advancing our understanding of the biological mechanisms involved in the stimulation of ovarian and testicular function via regulation of steroid hormone biosynthesis, gametogenesis and cell maturation. The pituitary derived gonadotropins, lutropin (LH) and follitropin (FSH), and the placental chorionic gonadotropin (CG), as well as the other pituitary glycoprotein thyrotropin (TSH) all share the distinctive features of two structurally dissimilar, non-covalently linked subunits, designated α and β[1,2]. The amino acid sequences of the α-subunit of these glycoprotein hormones are virtually identical within a given mammalian species with close structural homology between species. Considerable homology also exists across species for the amino acid sequences of the immunologically and biologically unique β-subunits which confer hormonal specificity. All the pituitary glycoprotein

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hormone α-subunits contain two oligosaccharide moeities N-linked to asparagine residues with 5 disulphide bonds, whilst the β-subunits contain up to two glycosylated asparagines, depending on the hormone, and 6 disulphide bonds. Subunit biosynthesis occurs by translation from separate mRNA species with a single gene coding for the common α-subunit and separate genes coding for the hormone specific β-subunits[3]. Combination of the α- and β-subunits is preceded by co-translational cleavage of the signal peptide and glycosylation by transfer of pre-assembled oligosaccharides from lipid carriers within the rough endoplasmic reticulum. Further processing of the glycosylated subunits to complex forms, such as sulphation by sulphotransferases, appears to occur in the smooth endoplasmic reticulum–Golgi membrane structure.

Since the processing of the subunit polypeptide and carbohydrate chains can be independently regulated by both endocrine and metabolic factors, this differential processing has important implications for modulation of hormone action in various physiological states. For example, evidence has been accumulated to show that the distribution of the isoforms of both LH and FSH in pituitary extracts and plasma from a number of species is influenced by age, gonadectomy, steroid hormone treatment or pathophysiological status. Biological studies[4–6] on the *in vivo* and *in vitro* bioactivities or the immunological and radioreceptor binding activities, of these glycoprotein hormones indicate that the isoforms are intrinsically different in their biological properties. Nearly all previous reports on the biological and immunological properties of these glycoprotein hormones have originated from preparations now known to be heterogeneous. In the present paper, we describe a new strategy for the rapid, high-resolution separation of microheterogeneous forms of these glycoprotein hormones from human pituitaries based on mild extraction conditions and combination of preparative isoelectric focusing and anion-exchange high-performance liquid chromatography (HPLC).

MATERIALS AND METHODS

International reference preparations (IRP)

The 2nd IRP for human pituitary LH and FSH for bioassay (code No. 78/549) and 2nd IRP for human pituitary TSH for bioassay (code No. 80/558) were provided by the WHO International Laboratory for Biological Standards and Control, London, U.K.

Hormone preparations

Highly purified hCG (CR121) was obtained from the NIADDK (Bethesda, MD, U.S.A.); hFSH Batch (81-1) 4150 IU/mg *in vitro* bioassay preparation and crude human pituitary FSH were provided by the Human Pituitary Advisory Committee (HPAC), Department of Health, Canberra, Australia. Highly purified hTSH (hTSH-Batch 1-5) and hTSH antiserum (Batch 3) were provided by the NIADDK and crude hCG (Pregnyl) preparations were obtained from Organon (Cambridge, U.K.).

Preparation of human pituitary extracts

Frozen human pituitaries, provided by the HPAC, were stored at −20°C prior

to the fractionation studies. The pituitaries were processed using the following procedures with extract operations carried out at 4°C. Fifty pituitary glands were thawed, minced finely and homogenized in 100 ml of 50 m*M* phosphate buffer pH 7.0 [195 ml of 0.2 *M* sodium dihydrogen orthophosphate, 305 ml of 0.2 *M* disodium hydrogen orthophosphate (anhydr.) per litre] for 5 min in a Polytron-Ultraturrax (Janke and Kunkel, Staufen, F.R.G.). The homogenate was then centrifuged at 100 000 *g* for 1 h and the supernatant recovered by gentle aspiration.

Gel chromatography on Sephacryl S200

The supernatant fraction containing the hLH, hFSH and hTSH activity was applied to a Sephacryl S200 (Pharmacia, Uppsala, Sweden) column (100 × 5 cm) and eluted with 0.05 *M* ammonium acetate, pH 7.0, at a flow-rate of 70 ml/h using a peristaltic pump (P-1, Pharmacia). Fractions of 8 ml were collected. The column fractions containing the hLH, hFSH and hTSH activity (fractions Nos. 10–58, volume 672–1064 ml) were pooled and lyophilised.

Preparative isoelectrofocusing in sucrose gradients

The lyophilisate was solubilized in water (Milli-Q, Millipore, Bedford, MA, U.S.A.), carrier ampholytes (pH range 3.5–10 Ampholine, LKB, Bromma, Sweden) were added and the solution centrifuged at 4300 *g*. The sample was electrofocused in a sucrose gradient with a 110-ml electrofocusing column (LKB) for approximately 20 h at 2000 V or 10 W at 4°C. The gradient was then eluted at 60 ml/h and 1.0 ml fractions collected. The pH was determined in every 5th tube using a pH meter (PHM 62 radiometer, Copenhagen, Denmark) attached to a chart recorder (Servograph REC 61 radiometer). The UV profile was monitored at 280 nm.

Preparative anion-exchange high-performance liquid chromatography

Equipment. A Pharmacia fast protein liquid chromatography (FPLC) system was used which consisted of two P-500 syringe pumps, a V-7 injector and a 278 nm fixed-wavelength single-path UV monitor, coupled to a two-channel REC 482 pen recorder. Gradient elution was controlled with a LCC-500 programmer. Mono-Q prepacked columns (HR 5/5, 50 × 5 mm I.D.) were used throughout. Column effluents were collected with a FRAC-100 fraction collector. All chromatographic separations were carried out at ambient temperatures (approx. 20°C). The flow-rate for the protein hormone isolations was 1.0 ml/min.

Buffers and samples. Piperazine, tris(hydroxymethyl)aminomethane (Tris) and bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane (Bis-Tris) were obtained from Sigma (St. Louis, MO, U.S.A.). Sodium chloride (Univar grade) was from Ajax (Sydney, Australia). Distilled water was further purified with a Milli-Q system (Millipore, Bedford, MA, U.S.A.). All buffers were filtered through 0.45 μm cellulose acetate filters (HAWP04700, Millipore). Buffer exchange of the appropriately pooled fractions, corresponding to *ca.* pH 0.2 increments of the preparative isoelectric focusing profile was carried out on PD-10 columns packed with Sephadex G-25 (Pharmacia), and pre-equilibrated with 20 m*M* piperazine, pH 9.6. The breakthrough peak, containing the hormonal bioactivities, was immediately loaded without concentration onto a Mono-Q anion exchange column also equilibrated in 20 m*M* piperazine, pH 9.6. Elution of proteins was achieved using a 0–300 m*M* sodium chloride linear gra-

dient, with a rate of change of [Cl^-] of 8.75 m*M*/min. The fraction size collected was 0.5 ml at a flow-rate of 1 ml/min. Similarly, the breakthrough peak from the PD-10 column containing all the hormonal bioactivity was loaded onto a new (or original) Mono-Q column freshly equilibrated with 20 m*M* Tris–HCl, pH 8.60 or 20 m*M* Tris–HCl, pH 7.6 or 20 m*M* Bis-Tris, pH 6.6 and elution again effected with a 0–300 m*M* sodium chloride gradient at 8.75 m*M* Cl^-/min.

Hormone assay procedures

hLH radioreceptor assay. The LH radioreceptor assay of Leidenberger and Reichert[7] was employed with the 2nd IRP for hLH and hFSH as standard and hCG (CR121), iodinated by a chloramine T procedure as tracer. The iodinated hormone was separated from ^{125}I by gel filtration with PD-10 columns equilibrated in 10 m*M* phosphate buffer containing 0.145 *M* sodium chloride, 0.15 m*M* Thiomersalate, 0.2% BSA pH 7.4 (0.2% BSA–PBS). Non-specific binding was assessed with a crude hCG preparation (10 IU Pregnyl). The membrane binding of [^{125}I]hCG in the assays ranged from 12 to 50%. The between-assay variation, as assessed from the repeated coefficient of variation of the analysis of a purified preparation, was 21.4%, $n = 36$. The within-assay variation, as assessed from mean index of precision (λ), was 0.045, $n = 36$.

hFSH radioreceptor assay. The FSH radioreceptor assay of Cheng[8] was used with the 2nd IRP for hLH and hFSH as standard and a highly purified hFSH preparation (Code No. 81/1) iodinated by the lactoperoxidase procedure of Thorell and Johansson[9] was used as tracer. The iodinated hormone was purified on an Ultrogel ACA 54 (LKB) column (90 × 1.6 cm) in 0.2% BSA–PBS pH 7.4. Non-specific binding was assessed ith a crude pituitary hFSH preparation provided by the HPAC. The membrane binding of [^{125}I]hFSH in the assays ranged from 5–26%, $n = 18$. The between-assay variation, as assessed from the coefficient of variation of the repeated analysis of a purified hFSH preparation, was 16.9%, $n = 14$. The within-assay variation, as assessed from the mean index of precision (λ), was 0.060, $n = 18$.

hTSH radioimmunoassay. A second antibody radioimmunoassay procedure was employed using reagents provided by the NIADDK with the 2nd IRP for human pituitary TSH for bioassay as standard, hTSH antiserum (Batch 3) and [^{125}I]hTSH (I-5) as tracer. The between-assay variation was 14.9%, $n = 16$ and the within assay variation (λ) was 0.056, $n = 16$.

Statistical analysis

Parallelism between standard and unknown preparations was established by 2-way analysis of variance of the slope values. The dose–response lines were linearized following log-dose transformation. In situations where non parallelism was observed between the dose–response line of the sample and that of the standard, a logit 0 value (equivalent to ED_{50}) of the unknown in terms of the standard was determined. Potency estimates of hLH, hTSH and hFSH were then determined using standard bioassay statistical designs.

RESULTS AND DISCUSSION

The presence of microheterogeneous forms of the gonadotropins and thyro-

tropins in biological extracts has been recognised for some time. The polydisperse nature of these glycoprotein hormones has been manifested not only in terms of differences in apparent molecular weight and charge, but also in the ratio of biological–immunological (B/I) activities. In a number of instances this microheterogeneity is clearly of clinical significance. Qualitative changes in gonadotropin secretion (mainly LH) are seen during puberty[10,11]. In the male, the LH secretion pattern changes with age, leading to the release of less biologically active forms[12]. In the female, significant difference in the molecular composition of the stored and circulating LH forms (as assessed by size exclusion chromatography or analytical isoelectric focusing) have been described[6,13] for women of fertile age and postmenopausal women. Nearly all of the preparations used to determine glycoprotein hormone activity in these and other studies were either unfractionated extracts, plasma or partially purified samples now known to be heterogeneous.

Recent advances in HPLC techniques offer one solution to resolution problems posed by these microheterogeneous glycoprotein hormones, and polydisperse proteins in general. As has been well documented in numerous conventional or HPLC studies related to the purification of proteins, the tandem use of two (or more) high-resolution chromatographic steps which exploit the largest selectivity differences offers the greatest potential for separating contaminating proteins from the desired product. Similar criteria apply to the resolution of a polydisperse protein into its various isoforms, although in this case more subtle manipulation of selectivity may be required. In several earlier studies, we have described[14–16] a generic approach to tandem HPLC separations which involve minimum sample handling or concentration prior to the final "polishing" stage. One aspect of this approach is the use of the same chromatographic media in several sequential steps with eluents of different composition to generate chromatographic selectivity.

Ion-exchange chromatography is well suited for such "selectivity modulation" by the eluent. Both the polyionic surface of the ion-exchanger and the surface charge characteristics of proteins are readily manipulated experimentally by the choice of pH, ionic strength or nature of the co- or counter ions, etc. Changes in gradient slope provide a further option to exploit band spacing and thus influence overall resolution[16]. This potential of ion-exchange HPLC gains even greater significance when it is recalled that a specific protein, irrespective of whether it is chromatographed on anion or cation exchangers, generally does not show minimum retention when the pH of the eluent corresponds to the iso-electric point (p*I*) of the protein in question. The p*I* of a protein is an important parameter in its physico-chemical characterisation and corresponds to the pH at which the protein has zero mobility, *i.e.* zero net charge, in an electrophoretic field. Similarly, "net charge" concepts have been widely used in the past to predict retention behaviour of proteins on ion-exchange resins. According to these concepts, retention correlates with net charge (Z) with the consequence that when the pH of the eluent–column equals the p*I* of a protein then it will show no retention (*i.e.* k' and Z are both zero). When the pH of the eluent–column is above the protein's p*I* value it will have a net negative charge and be retained on an anion-exchange column whilst if the eluent–column pH is below the protein's p*I* value, the protein will be positively charged and only be retained on a cation-exchange column. However, the "net charge" hypothesis does not take into account charge asymmetry, the involvement of ion-bridges or other coulombic features which

constitute part of the protein's topography. The retention map (relative retention *versus* pH) of a protein with, say, a p*I* 8.0 value, would thus be expected in general not to experimentally follow the hypothetical shape as shown in Fig. 1a but more usually to take the form of Fig. 1b. If the protein exists as several different isoforms, each having the same p*I* (and even the same net charge when pH ≠ p*I*), chromatographic resolution of these isoforms is still theoretically feasible provided surface charge asymmetry exists between the different isoforms. This hypothesis has been tested as part of our ongoing studies on the purification of hLH, hFSH and hTSH from pituitary extracts and other biological sources.

Fig. 2 shows the optical density profile and the hormone bioactivity profiles for hLH, hFSH and hTSH following preparative isoelectric focusing of a human pituitary protein fraction on sucrose density gradients. The extensive heterogeneity of these glycoprotein hormones with respect to p*I* is clearly evident from this figure. If a narrow zone of these proteins with the same apparent p*I* value is subsequently chromatographed on a strong anion exchanger, such as Mono Q, then further frac-

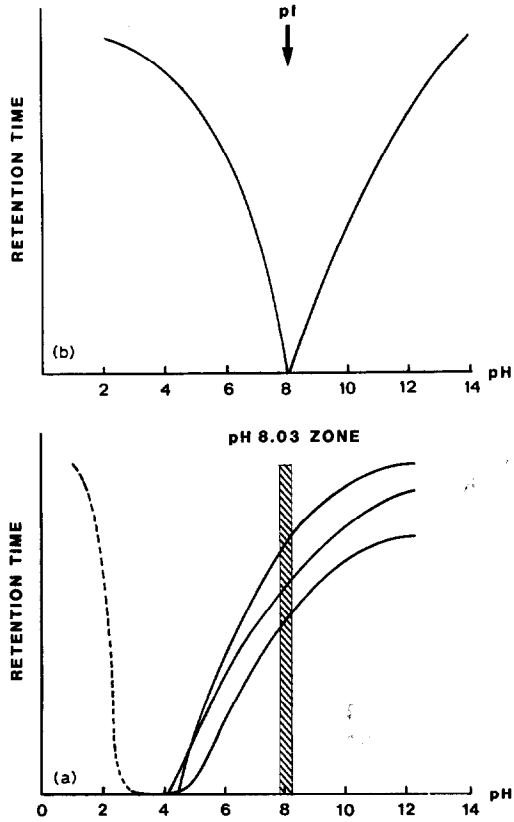

Fig. 1. (a) Hypothetical plot of relative retention versus eluent pH for a protein with a p*I* 8.0 according to the "net charge" hypothesis. (b) Hypothetical plot of relative retention versus eluent pH for three proteins (or three protein isoforms), all of which exhibit a p*I* 8.0, according to the "charge asymmetry" hypothesis.

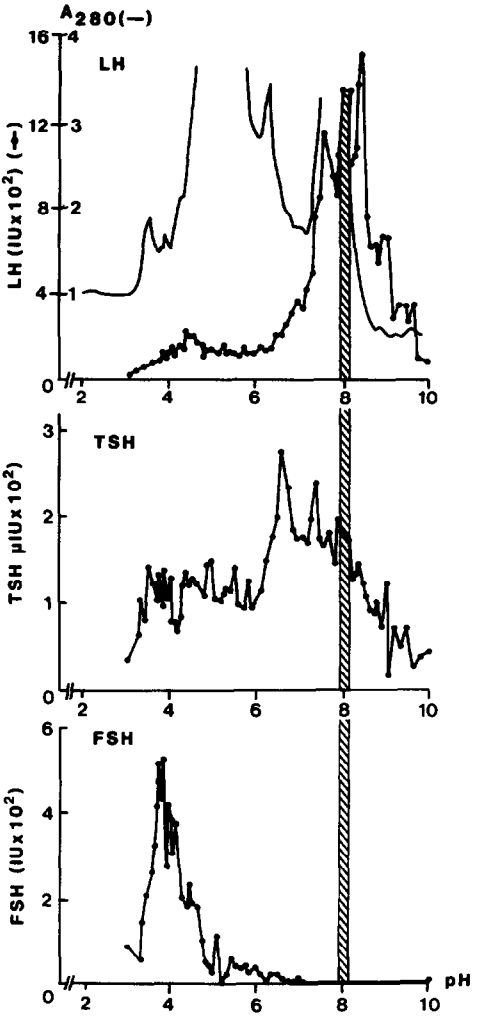

Fig. 2. Optical density profile and hormone bioactivity profile for hLH, hTSH and hFSH following preparative isoelectric focusing of a human pituitary fraction on a sucrose density gradient. The hatched zone, corresponding to proteins with an apparent p*I* 8.0, was employed in the subsequent experiments illustrated in Figs. 3–5.

tionation of these proteins can be achieved. Fig. 3 illustrates a typical chromatographic example in which the hLH and hTSH isoforms, contained in the narrow protein zone corresponding to pH 8.03 ± 0.16 of the preparative isoelectric focusing experiment, are further resolved such that with a 20 m*M* piperazine–sodium chloride, pH 9.6, eluent at least 3 hLH and 4 hTSH isoforms can be discriminated. Moreover, if the same chromatographic media is used with a 20 m*M* Tris–HCl, 0–300 m*M* sodium chloride, pH 7.6, eluent a dramatic change in selectivity between the hLH and hTSH isoforms occurs (Fig. 4). The preferential movement of the LH and TSH isoforms with respect to each other, and the overall reduction in protein retention

behaviour, is evident by comparison of data shown in Figs. 3 and 4. The change in chromatographic selectivity for the three hLH and the four hTSH isoforms, all of which exhibit an apparently common isoelectric point of *ca.* p*I* 8.0, as a function of the eluent pH is shown in Fig. 5. As is evident from these retention maps, significant selectivity changes for these glycoprotein hormones can be achieved with eluents encompassing relatively narrow pH differences. These observations are in accord with the conclusion that relative retention of proteins on ion exchange supports is strongly influenced by the spatial arrangement of the charges on the protein surface, in addition to the net effective charge *i.e.* differences in chromatographic selectivity with ion-exchange HPLC supports for closely related proteins is dependent on charge asymmetry differences between the protein isoforms. Similar behaviour has been noted[16,17] with other native and chemically modified globular proteins. The above

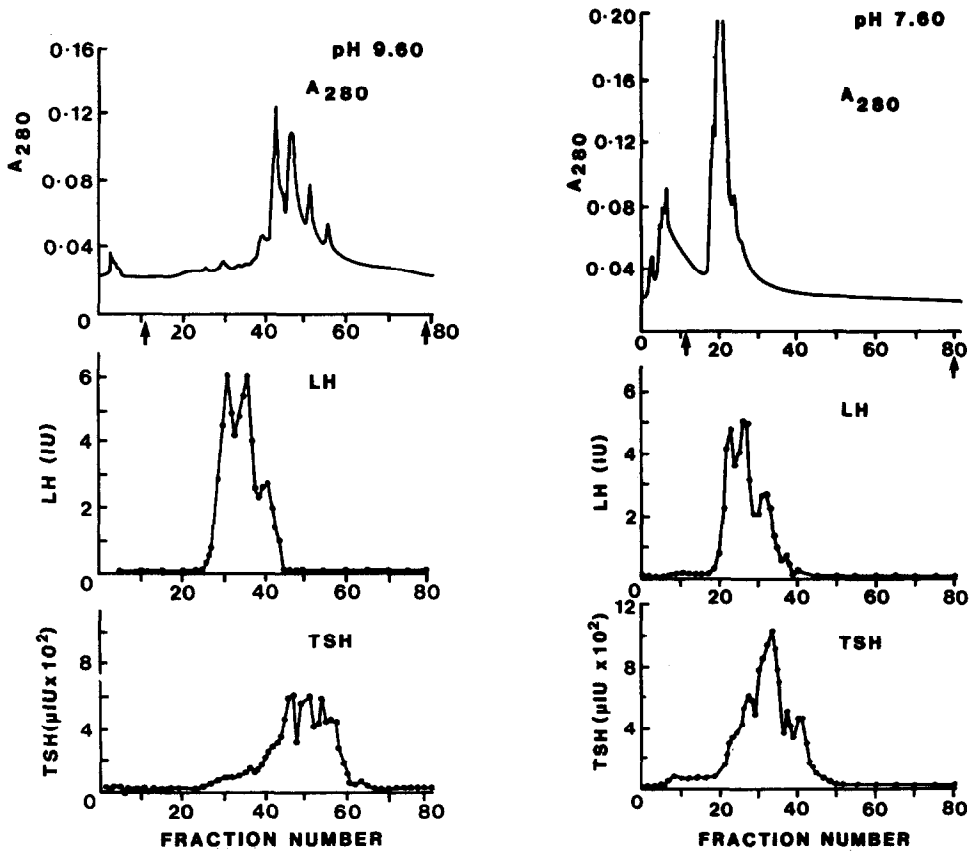

Fig. 3. Typical chromatographic profile and hLH and hTSH bioactivity profile when a narrow p*I* zone (p*I* 8.03) of proteins are chromatographed on a Mono Q column with a 20 m*M* piperazine, pH 9.6 buffer and a 0–300 m*M* sodium chloride linear gradient. The arrows indicate the initiation and completion of the gradient.

Fig. 4. Typical chromatographic profile and hLH and hTSH bioactivity profile when a narrow p*I* zone (p*I* 8.03) or proteins are chromatographed on a Mono Q column with a 20 m*M* Tris–HCl, pH 7.6, buffer and a 0–300 m*M* sodium chloride linear gradient.

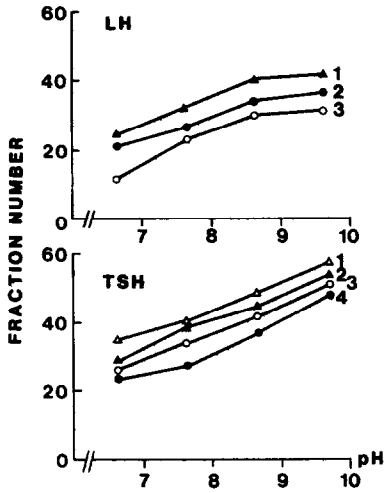

Fig. 5. Retention maps for the three hLH isoforms and the four hTSH isoforms, all of which exhibit an apparent p*I* 8.0 in isoelectric focusing experiments. The experimental data is plotted as fraction number (= elution volume) versus eluent pH.

results are also in accord with an earlier report[18] which also documented similar extensive hormone heterogeneity with a purified hLH preparation.

Potentially a very large number of isoforms could arise with a particular glycoprotein hormone due to post translational modifications to the carbohydrate sidechains and the peptide backbone including sulphation, phosphorylation and deamidation. Although the above experiments largely correspond to separations at the analytical (*i.e.* 100 μg) level, the results nevertheless validate the potential for "selectivity modulation" in ion-exchange HPLC. In this study the same ion-exchange stationary phase is used in tandem steps with eluents of different ionic composition and pH. Clearly other scenarios are equally feasible, *i.e.* mixed bed approaches. Conceptually, these approaches bear close similarities to the selectivity strategies now well documented for ion-pair reversed phase HPLC of polypeptides and proteins[19]. By appropriate combination of two or more high-resolution tandem steps, purification of a particular set of isoforms by rapid chromatographic procedures can be undertaken. In an associated paper the application of these concepts to the preparative recovery of an isoform set of human LH and TSH, essentially free of other protein components, is described.

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