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REVIEW

SEPARATION OF PROTEIN HORMONES

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CONTENTS

1. INTRODUCTION

The scope of this review is to present modern methods for protein hormone separations. Since the early 1980s various techniques such as paper chromato-

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graphy, liquid-phase extraction, and soft-gel column chromatography have been rapidly supplanted by high-performance liquid chromatography (HPLC) because of superior separation with better reproducibility and shorter analysis times. This discussion will concentrate on the various HPLC columns, mobile phases, and sample preparation procedures used for the separation and analysis of ten selected protein hormones. These macromolecules range from the well known insulin molecule to analytes that have recently become clinically significant such as atrial natriuretic peptides and human growth hormone (hGH). We purposely chose a size array from the five amino acid residue enkephalins to much larger molecules, such as β-lipotropin, insulin, and parathyroid hormone, to present inherent problems in analysis due to size. For example, analysis of larger molecules is more readily affected by proteases, conformational changes, less efficient extraction, and immunospecificity problems related to sample heterogeneity. Small peptides on the other hand are more difficult to separate away from buffer salts and cross-immunoreactivity of precursor forms can present significant errors in quantitation. The ten protein hormones selected for this review may not necessarily be the most clinically important ones, but the collected strategies for their separation, detection, and analysis should provide the researcher with several practical tips for analysis of most protein hormones in biological fluids or tissues.

Since it has been our practical experience in the discovery, isolation, and characterization of proteins that electrophoretic analysis is an essential complement to HPLC in the protein hormone research laboratory, we have included a section on this topic. Special emphasis is placed on two-dimensional (2-D) gel analysis because of unique selectivities that are not possible by any HPLC mode. While not suggested as a replacement for clinical analyses readily done by HPLC, 2-D gel analysis can provide insight leading to the identification of new protein hormones or analogues of known structures which may be clinically important.

2. HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF PROTEIN HORMONES

2.1. Sample handling

Proteins from tissue homogenates, plasma, urine, or other sources can be fractionated by either sodium sulfate precipitation or acid extraction. Regardless of initial fractionation it is advisable to add enzyme inhibitors such as phenylmethylsulfonyl fluoride (PMSF) at about 10 μg/l to inhibit serine proteinases and pepstatin A at 1–10 mg/l to inhibit carboxyl proteinases. Additional PMSF may be required during subsequent purification steps since its half-life in extract is reduced under conditions such as slightly alkaline pH or warming to room temperature [1]. Other inhibitors such as the broad-spectrum inhibitor leupeptin (1 mg/l), metalloprotease inhibitor ethylenediaminetetraacetic acid (EDTA; 1 mM), leucine aminopeptidase inhibitor bestatin, and plasmin inhibitor benzamidine (50 mM) may be advisable depending on the sample source. For example, benzamidine is especially useful for whole blood samples because it is an inexpensive competitive inhibitor of thrombin, trypsin, and plasmin while being non-immunoreactive. Caution should be exercised when using polypeptide-based

inhibitors because of possible immunoreactive or immunomodulatory assay interference. After choice of suitable protease inhibitors, preparations are usually acid-extracted with 1–3 *M* acetic acid followed by centrifugation. Other acids commonly used for extraction include 2–10% formic acid, 1–2 *M* hydrochloric acid, and 1–2% trifluoroacetic acid (TFA). If large proteins are not important they can be precipitated out with a simultaneous delipidation step by dichloromethane extraction. Trace amounts of dichloromethane in the aqueous phase are then removed by aspirator vacuum. Trichloroacetic acid (TCA) protein precipitation can also be used for selective precipitation and subsequent resuspension. The next step in a typical purification is immunoaffinity chromatography if an antibody is available, followed by gel permeation chromatography (GPC) in 2–5 *M* acetic acid. Until recently it has been common practice to lyophilize the GPC pools for storage prior to HPLC. Laboratories now have begun diluting GPC eluents with 0.1% TFA and pumping them directly onto reversed-phase HPLC (RP-HPLC) columns. This simple procedure allows the salt to wash through the column. Alternatively, if HPLC analysis will not immediately follow or if a preparative batch of extract is being desalted, this procedure can be done on a solid-phase extraction cartridge such as a Sep-Pak (Waters Assoc., Milford, MA, U.S.A.) with subsequent lyophilization and storage for future HPLC analysis.

2.2. Column and mobile phase selection

The most important trick to HPLC analysis is picking the right column before spending a great deal of time optimizing the mobile phase. The best resolution is usually obtained by the reversed-phase mode. It has been our experience that recoveries are higher and resolution is better for proteins when C_4 or C_8 as opposed to C_{18} alkyl phases are used [2]. Smaller polypeptides in general (less than 20–30 residues) do not have this recovery problem unless they are extremely hydrophobic. Since HPLC columns are expensive, variety in the matrix (type of silica or synthetic organic resin) as opposed to different alkyl chain lengths on the same support is favored. As previously mentioned, alkyl chain length may influence recovery but in general it has been our experience that columns with various chain lengths from the same manufacturer give only slightly different selectivities. Altered selectivity is better achieved by going from an alkyl chain to a diphenyl or cyanopropyl phase. Diphenyl phases may give selectivity difference by virtue of π–π interactions with aromatic groups, while cyanopropyl phases have been especially favored for glycopeptide separations. Large polypeptides (greater than 30–40 residues) and proteins tend to separate better on macroporous supports. The support material we routinely use in this class is Vydac (330-Å pores) from the Separations Group (Hesperia, CA, U.S.A.). It is widely available although usually sold under different brand names in pre-packaged columns. Smaller peptides also separate well with this material, but since they also separate well on many smaller-pore materials (60–150 Å) that have inherently higher surface areas, peptide loading capacity can be much greater on these smaller-pore-sized materials. The C_{18} μBondapak column (Waters Assoc.) works very well for smaller peptides and has been used by many investigators.

After assessing the limits of RP-HPLC for a separation, cation- and anion-exchange HPLC should be used next. If the protein hormone isoelectric point (p*I*) is unknown, it is statistically best to start with an anion exchanger because two thirds of known protein p*I* values would favor this mode while only one third would be suited for cation-exchange HPLC. There are far fewer ion-exchange HPLC columns on the market than for RP-HPLC. Our experience has been that the polyethyleneimine-coated supports from Synchrom (Linden, IN, U.S.A.), Pharmacia (Piscataway, NJ, U.S.A.), and the TSK-DEAE columns distributed for Toya Soda (Japan) under various names work very well. The Polyaspartic acid and Polysulfoethyl cation-exchange columns based on 330-Å silica by Poly LC (Columbia, MD, U.S.A.) work well for protein and peptide separations, respectively. Hydrophobic-interaction chromatography (HIC) is a new mode recently introduced by column manufacturers. Proteins in this case are eluted by a descending salt gradient; this mode may be useful for purification of surface hydrophobic and/or labile proteins.

Mobile phases commonly used for soft-gel ion exchange work reasonably well in HPLC-based methods, but care has to be taken when using corrosive salts. Flushing the system with plenty of water at the end of the day is advisable. While there are hundreds of commercially available RP-HPLC columns to choose from, fortunately only a handful of mobile phase systems need to be tried. The most popular uses 0.1% (v/v) TFA as an ion-pairing agent (IPA) with gradients up to 60–80% acetonitrile or 40–60% propanol over 20–80 min at 0.5–1.5 ml/min for 4.6 mm I.D. columns. Increasing the concentration of TFA to 0.2–0.3% will increase retention time and may help to slightly alter selectivity. Heptafluorobutyric acid (HFBA) may also be used and gives greater retention than TFA but is not as readily volatile. Other IPA alternatives include non-volatile 0.155 *M* sodium chloride (adjusted to pH 2 with hydrochloric acid), 0.1 *M* phosphate at pH 2, or higher-pH mobile phases containing ammonium bicarbonate or acetate.

2.3. Detection

Detection of peptides is commonly done in the UV range 210–230 nm depending on background interference, while peptides with aromatic groups and proteins are monitored at 280 nm. Fluorescence detection is more sensitive but requires post-column derivatization and stream splitting if sample needs to be recovered. In the clinical setting detection of trace quantities of protein hormones from biological samples is typically done by radioimmunoassay (RIA) or radioreceptor-assay (RRA). Several examples of these immuno-based detection techniques and a discussion of their differences are given in Section 2.4.

2.4. High-performance liquid chromatographic analysis

2.4.1. Adrenocorticotropic hormone-related peptides

Adrenocorticotropic hormone (ACTH) is a peptide which regulates the growth and function of the adrenal cortex and controls the secretion of cortisol and dehydroepiandrostone from the fasciculata–reticularis of the adrenal cortex. ACTH

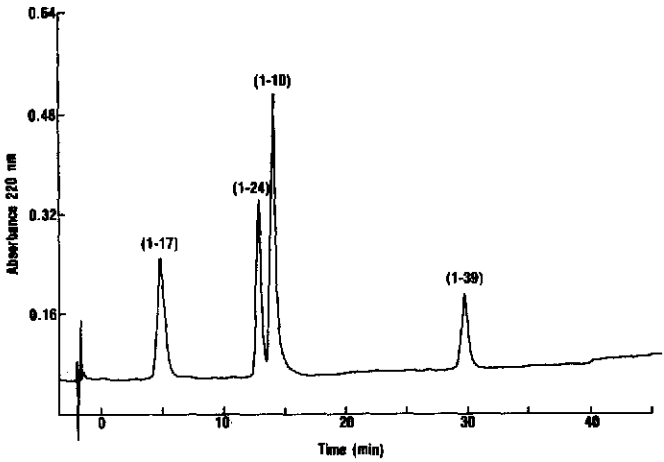

Fig. 1. Separation of human ACTH and fragments 1-10, 1-17, and 1-24. Column, Vydac 25 cm×4.6 mm C_8 (228TP7415); buffer A, 0.08% (v/v) TFA; buffer B, 0.08% (v/v) TFA in 80% acetonitrile; gradient, 20 to 50% B in 60 min; sample, 1.25 μg peptide; flow-rate, 1 ml/min; detection, 220 nm, 0.64 a.u.f.s.; temperature, ambient.

also stimulates glucose uptake and inhibition of pyruvate transport in the adipocyte [3]. It is a straight-chain polypeptide containing 39 amino acids and has a relative molecular mass (M_r) of 4500. Mammalian ACTHs are highly conserved varying in only two amino acids between species. The active part of the peptide causing adrenal glucocorticosteroid release is found in the first 18–24 amino acids. The carboxy terminal corticotropin-like intermediate peptide (CLIP) fragment 18-39 contributes minimally to its biological activity.

Like most peptide hormones, ACTH is synthesized as a pro-precursor. ACTH and β-lipotropin (β-LPH) share a common precursor protein which is enzymatically cleaved into ACTH, β-LPH, and a biologically inactive 16-kD fragment. In turn, ACTH is cleaved to form α-melanocyte stimulating hormone (α-MSH) and β-LPH is cleaved to form the endorphins.

Many studies have employed HPLC to isolate and/or separate ACTHs in biological and synthetic preparations. Burgess and Rivier [4] in one of the earliest papers on peptide HPLC used 0.01 *M* ammonium acetate buffer (pH 4.2) with and without 1–5% acetic acid on a reversed-phase column to resolve ACTHs from α-MSHs. Bennett et al. [5] have since published a procedure with more volatile ion-pairing reagents in which they were able to isolate and purify two major forms of ACTH from rat pituitary. This paper also discusses the superiority of HFBA over TFA as the stronger ion-pairing reagent for hydrophobic basic peptides. Their system used a Waters μBondapak C_{18} column with mobile phases of either 0.1% TFA or 0.13% HFBA eluted by a linear gradient of acetonitrile. A sample chromatogram from our laboratory run with slightly different conditions is shown in Fig. 1. This general separation methodology coupled with a commercial ACTH RIA kit for assay of subsequent fractions can be set up very easily. As illustrated in Fig. 1 the order of peptide elution is not dependent on chain length. In fact,

both relative hydrophobicity of individual residues and tertiary structure contribute to peptide retention on the reversed-phase column. It is beyond this review to include relevant papers on peptide retention time prediction; the reader is therefore referred to another review discussing this issue [6].

An extensive review of the literature concerning the use of HPLC to separate various ACTHs was published by Verhoef et al. [7] and serves as an excellent reference guide for mobile phases if the chromatographer is interested in isolating the numerous post-translational modified ACTH species.

2.4.2. Angiotensins

The renin–angiotensin system is a clinically important biochemical pathway affecting vasoconstriction and thereby regulating blood pressure. The sequence of events begins with the amino terminus of angiotensinogen being enzymatically cleaved by the endopeptidase renin to produce angiotensin I (Ang I). A peptidyldipeptidase then converts angiotensin I to the more biologically active octapeptide angiotensin II (Ang II) [8], causing increased blood pressure. An aminopeptidase can remove the amino terminal aspartic acid of Ang II to yield the less active angiotensin III (Ang III) [9]. Angiotensin-converting enzyme inhibitors such as Captopril inhibit metabolism of Ang I to Ang II and are currently used to control hypertension by modulating these biochemical events. The tools of biotechnology have recently allowed great advances in the quest for renin inhibitor peptides to control Ang I production now that recombinant human renin has been cloned, expressed, and purified [10]. It is therefore important to clinically assay a variety of angiotensin-related peptides.

Human arterial plasma Ang II immunoreactivity is 10–50 fmol/ml [11] with RIA sensitivity limits to 5–10 fmol [12–14], but direct RIA of Ang II in blood samples is unreliable because of cross-reactive precursors and/or metabolites. Therefore HPLC is necessary to isolate these post-translationally modified species. Purified fractions can then be assayed by RIA; this is necessary because flow-through HPLC detectors (UV, post-column fluorescence, and electrochemical) are not nearly sensitive enough to measure femtomolar amounts of biologic sample.

Angiotensin peptides have been effectively separated by a variety of chromatographic techniques. Early separations were achieved using paper and thin-layer chromatography (TLC) [15, 16] but they were time-consuming. RP-HPLC and ion-exchange HPLC techniques for resolving angiotensins are now abundant and the method of choice. Several good examples are found in the literature. Molnár and Horváth [17] separated Ang I and Ang II from angiotensin peptide fragments on a LiChrosorb RP-8 reversed-phase column using an acetonitrile gradient in a phosphate buffer. Although this method was published nearly ten years ago, the demonstrated selectivity is comparable to newer methods. The only disadvantage is that the system uses a 70 °C temperature which significantly reduces column lifetime. Resolution of commercial angiotensin from closely related isomers, diastereomers, and other impurities was recently achieved with a triethylamine phosphate (TEAP)-buffered acetonitrile gradient at room temperature on a C_{18} column [18]. Still another excellent example (Fig. 2) for a 20-min isocratic resolution of closely related angiotensins down to the 10-pmol level employs

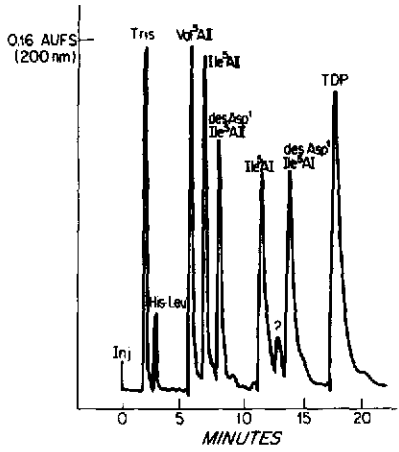

Fig. 2. Separation of angiotensin peptides by RP-HPLC. Samples, 1 nmol each of [Ile^5]Ang II, des[Asp^1]-[Ile^5]Ang II, [Val^5]Ang II, and His-Leu; 2 nmol each of [Ile^5]Ang I and des[Asp^1]-[Ile^5]Ang I; and 3 nmol of NH_2-terminal tetradecapeptide of equine angiotensinogen (TDP). Mobile phase, 0.017 *M* phosphoric acid, 0.1 *M* sodium perchlorate in acetonitrile–water (38:62, v/v) at 1.0 ml/min isocratic flow; column, Ultrasphere ODS 250 mm × 4.6 mm with 25 mm × 4.6 mm precolumn. Tris added to sample to mark column void volume. Reprinted from ref. 19 with permission.

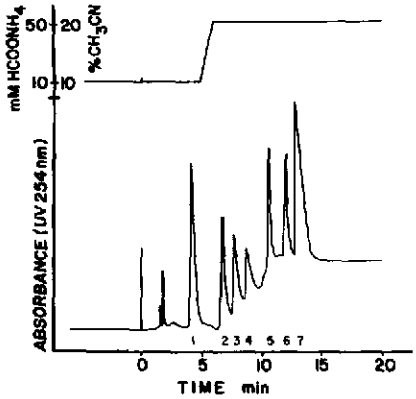

Fig. 3. Separation of seven angiotensin peptides (25 pmol each) using gradients of salt strength and acetonitrile concentration over a weak cation-exchange column (5 μm, 25 cm × 4.6 mm Analytichem CBA). Flow-rate, 2 ml/min; pH 4.2. Peaks: 1 = angiotensin C-terminal tetrapeptide; 2 = angiotensin C-terminal pentapeptide; 3 = Ang II; 4 = angiotensin C-terminal hexapeptide; 5 = Ang III; 6 = Ang I; 7 = des[Asp^1]Ang I. Reprinted from ref. 21 with permission.

perchlorate plus phosphate in ion-pair RP-HPLC [19]. Comparing the above three IPAs in the clinical setting it must be remembered that TEAP preparation requires more handling on a routine basis compared to phosphate/perchlorate systems.

The utility and effectiveness of weak anion-exchange HPLC was demonstrated by Dizdaroglu et al. [20] for the separation of complicated angiotensin mixtures with 90–98% recoveries. They used a Micro-Pak AX-10 column (Varian, Walnut Creek, CA, U.S.A.) with an acetonitrile gradient in TEAP buffer. Depending upon what other analytes are in a biological sample or synthetic mix, weak cation-

exchange HPLC can also be used to selectively retain and resolve angiotensins. Doris [21] accomplished resolution of up to seven synthetic angiotensin peptides at the 25-pmol level using an acetonitrile gradient in an ammonium formate-buffered (pH 4.2) mobile phase (Fig. 3). This separation was done on a weak cation exchanger with carboxylic acid functional sites and illustrates an alternative to alkyl-ligand bonded RP-HPLC. Although at pH 4.2 angiotensins have a net positive charge, this system is not a pure ion-exchange separation because the addition of organic solvent to the gradient was required to optimize the selectivity. It can therefore be best described as cation-exchange–hydrophobic-interaction or mixed-mode separation.

In summary, excellent separations of angiotensin peptides can be accomplished by several HPLC modes. The investigator must select the system most easily applied to his or her circumstances. That is, are the volatile buffer systems of reversed-phase techniques necessary, or are the near neutral mobile phases of ion-exchange desirable? In general, completely volatile reversed-phase systems require less HPLC hardware maintenance than non-volatile salt mobile phases for routine assay. On the other hand, in purification procedures ion-exchange salt gradients are often used in initial fractionation steps prior to desalting and subsequent RP-HPLC analysis.

2.4.3. Atrial natriuretic factor

Atrial natriuretic factor (ANF) was discovered in 1981 by De Bold et al. [22]. Scientifically this hormone is a significant find because it is produced and secreted from the heart, circulates in plasma, and binds to receptor sites on other organs, thereby establishing the heart as an endocrine organ. ANF is a 28-amino acid polypeptide with one disulfide bridge which is essential for maintaining biological activity [23]. Several immunoreactive ANF species in the range of 20–40 amino acid residues have been reported by researchers, but they are closely related structures usually differing by proteolytic effects during purification and probably all are related to the C-terminal region of ANF propeptide [24–26]. The amino acid sequence of the ANF precursor has been established from the cloned complementary deoxyribonucleic acid (cDNA) sequence of messenger ribonucleic acid (mRNA) [27, 28]. The known biological activities of ANF include lowering blood pressure, inhibiting aldosterone biosynthesis, promoting natriuresis and diuresis, relaxing adrenergic or cholinergic agonist-induced smooth muscle contraction, and possibly inhibiting renal secretion of renin. It is an exciting possibility that ANF may be part of a system counter-balancing the renin–angiotensin system (see Section 2.4.2.). ANF assays may be important for assessing primary or secondary hypertension, and excellent reviews have been written on its function [29–31].

ANF structure is highly homologous in mammals. Human, bovine, and porcine species are identical, with rat and mouse both having only an Ile 12 substitution for Met13 [32]. The RIA detection limit for ANF in human plasma is about 1.8 pg/ml [33, 34] for normotensive subjects having 30–65 pg/ml levels [33–35]; hypertensive patients have seven times that amount [34]. Interestingly, ANF

levels can increase by 150% if blood is drawn from humans in a prone position for 5 min as opposed to an upright position.

Procedures for RIA of ANF have been reported for atria [35–37], brain [36, 38], and plasma [24, 36]. Laros et al. [39] have suggested an improvement in the general procedure that increased recovery. Using C_{18} silica extraction cartridges coated with triethylamine acetate pH 4, recoveries greater than 78% were achieved by eluting with 80% methanol in acidic buffer.

A very efficient procedure for ANF purification from bovine atria has been reported by Ong et al. [32]. Atrial appendages were finely minced, polytroned, and passed over carboxymethyl agarose. The extract was then dried, resuspended in 2 *M* acetic acid and applied to a Sephadex G-50 column (Fig. 4a). Fractions with ANF immunoreactivity were then passed through a series of four HPLC columns. First, on a sulfopropyl cation-exchange column (Fig. 4b), then on a reversed-phase Ultrapore RPSC column (Fig. 4c), followed by a Vydac 218 TP54 (C_{18}) reversed-phase column (Fig. 4d), and finally on a μBondapak column (Fig. 4e). Although purification schemes such as this are determined by trial and error, a few points of logical progression should be noted. GPC and ion-exchange steps are usually done prior to reversed-phase chromatography because the salts used for separation can be easily extracted by charging the peptides onto reversed phases [40]. Then by successive changes in the stationary phase matrix, different selectivities can be achieved [41]. Both TFA and HFBA are excellent volatile ion-pairing reagents, but note that the final reversed-phase gradient uses TFA and not HFBA (Fig. 4); this is because TFA has a sharply lower boiling point which makes it easier to completely remove.

Other HPLC procedures are available for ANF identification and purification which have different column selectivities. For example size-exclusion chromatography (SEC) on a TSK-125 column prior to reversed-phase chromatography can be used [34]. Completely different columns such as DEAE-cellulose, followed by Zorbax propylcyano and Vydac phenyl HPLC bonded phases work effectively for ANF identification and purification [23].

2.4.4. Calcitonin

The first reference to calcitonin (CT) activity in the literature appeared in 1961 where Copp et al. [42] reported on a new parathyroid hormone which lowered blood calcium. It is now established that CT is a 32-amino acid peptide important in maintaining calcium homeostatis. Clinically, elevations of plasma CT are associated with medullary thyroid carcinoma (MCT) [43], renal failure, and hypercalcemia [44, 45]. CT RIA can be used to screen families for premetastasizing C-cell hyperplasia [46], a harbinger to MCT. It has also been postulated that CT itself protects against uremic [47] and postmenopausal bone loss [48].

RIA detection of CT has a lower limit of about 20 fg/ml [49], with normal human CT levels being in the 35–135 fg/ml range. Higher values may result from non-specific CT-like materials or CT precursors. This immunochemical heterogeneity is evident during GPC [50] and isoelectric focusing (IEF) [51] proce-

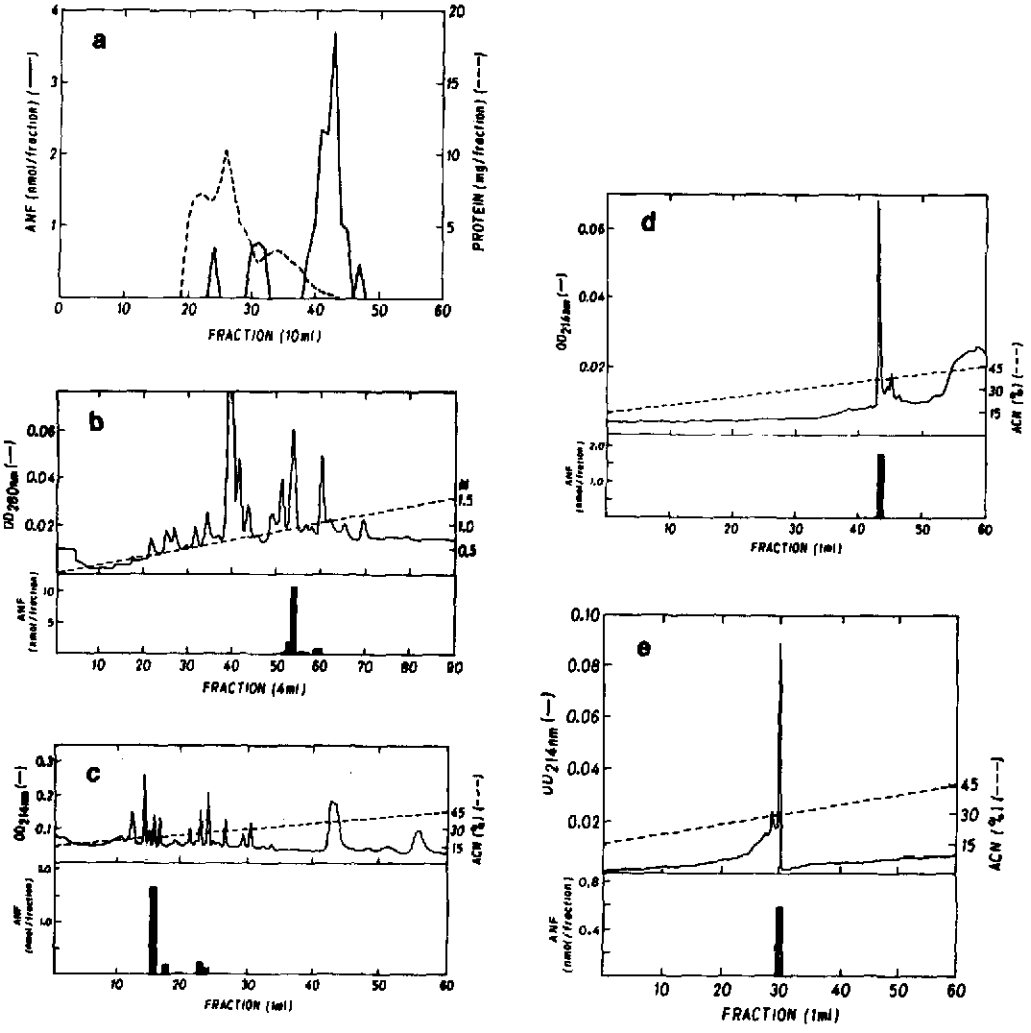

Fig. 4. (a) GPC on Sephadex G-50 (100 cm×2.5 cm) of the active ANF fractions eluted from a carboxymethyl Bio Gel A column. Elution was performed with 2% (v/v) triethylamine at a flow-rate of 0.5 ml/min. (b) Cation-exchange HPLC on sulfopropyl Protein Pak SP-5PW (21.5 mm×150 mm). The pooled active fractions from a were applied and eluted with a gradient from 0 to 1.5 *M* ammonium acetate in 0.5 *M* acetic acid at a flow-rate of 4 ml/min. (c) HPLC separation of the active fractions from b on an Ultrapore RPSC column (75 mm×4.6 mm). Elution was done by a linear gradient of 15 to 45% acetonitrile (ACN) in 0.1% TFA over 60 min at a flow-rate of 1 ml/min. (d) Rechromatography of the active material from c on a Vydac C_{18} 218TP54 column (250 mm×4.6 mm) using an acetonitrile gradient from 15 to 45% in 0.05% HFBA over 60 min at a flow-rate of 1 ml/min. (e) Final purification of the active fractions on a μBondapak C_{18} column (300 mm×3.9 mm) with a linear gradient of acetonitrile from 15 to 45% in 0.1% TFA over 60 min at a flow-rate of 1 ml/min. Reprinted from ref. 32 with permission.

dures where several discrete pools of immunoreactivity can be identified. MCT tissue contains 100 to 10 000 times more CT than normal thyroid glands [52].

Classical separation and purification of CT using GPC, cation-exchange chro-

matography, and electrophoresis give low yields of immunoreactive peptide and are quite time-consuming. Final steps of CT isolation from thyroid extracts of serum can be replaced by RP-HPLC. This straightforward method includes tetrabutylammonium phosphate or sodium sulfonyl hexane alkyl IPAs with methanol gradients on a μBondapak C_{18} column [53] or, preferably, HFBA with acetonitrile gradients on Nucleosil C_{18} [52, 54].

2.4.5. Cholecystokinins

Cholecystokinin (CCK) was first isolated in 1964 as a 33-amino acid polypeptide from porcine intestine [55]. It is now known that CCK-related peptides are a series of homologous peptides 4–39 amino acids in length [56]; this has been confirmed by cDNA sequence analysis of preprocholecystokin (pre-pro CCK) [57]. The C-terminal octapeptide CCK_{32-39} (CCK-8) is thought to be the most active form based on receptor binding and other studies [58], although human studies indicate CCK-33 and CCK-39 may also be important [59]. CCK activities include gallbladder contraction [60], pancreatic enzyme secretion [61], central nervous system (CNS), neurotransmission [62–64], and possibly plays a role in schizophrenia [65–67].

CCK is found in both brain and gut, where different post-translational cleavages occur. Rat pre-pro CCK is about M_r 13 000 and contains a single CCK-33 sequence [57]. Both CCK-33 and CCK-8 end with C-terminal phenylalanine amides, which are necessary for biological activity. CCK receptors are found in both pancreas and brain [68]. Pancreatic receptors are characterized as having high affinity for the CCK-8 sequence with a sulfated tyrosine seven residues from the C-terminus, whereas the CCK brain receptors are not that specific [58]. In the brain, the sulfated CCK-8 is most abundant [62, 69], while larger forms are more abundant in the intestine [70].

RIA of CCKs is difficult because so many forms exist and anti-CCK antibodies also cross-react with gastrins. Therefore HPLC procedures have to be used to separate the various CCKs (4, 8, 12, 33, and 40 amino acid residues and their post-translational derivatives) before proceeding with a RIA. Some HPLC systems that have been used include tandem μBondapak C_{18} and phenyl columns with TFA and acetonitrile gradient [71]; a μBondapak C_{18} column alone with pH 5.1 ammonium acetate as the IPA [58], and the more conventional TFA [70, 72] or HFBA [56] systems on reversed-phase columns with acetonitrile gradients. Maton et al. [59] have outlined a procedure for isolating individual CCKs from plasma samples. This was done on a Merck LiChroprep column with 155 m*M* sodium chloride and a linear acetonitrile gradient. Mean total serum CCK increased from <1 to 21 pmol/l for fasted humans after eating, with peak levels of CCK-33 and CCK-39 occurring later than for CCK-8. Other laboratories have reported higher total CCK levels without doing HPLC steps to separate individual CCKs [73, 74]. These higher levels may include interfering species or, on the other hand, additional HPLC manipulations may decrease total recovery of the individual CCKs. Additional studies must be done to establish not only total CCK levels, but also biological levels of individual species, and correlate these results with clinical observations.

2.4.6. Enkephalins

Methionine enkephalin (ME) and leucine enkephalin (LE) are naturally occurring peptides first discovered in the brain [75] and later in various peripheral tissues [76, 77]. These endogenous pentapeptide sequences mainly recognize δ-receptors [78] and differ only in their carboxy-terminal amino acid sequences (YGGFM versus YGGFL). ME and LE are part of a general class of opioid peptides (OPs) which are found along with catecholamines in purified chromaffin granules from the adrenal medulla [79] and in splenic nerve [80]. Timmers et al. [81] have shown that ME and LE are also in the pancreas [82], primarily localized in the islets of Langerhans. Extracts secreted into the circulatory system from phaeochromocytomas have also been found to contain LE, ME, and related enkephalins along with their large-molecular-mass precursors [83, 84]. Like other regulatory peptide fields, the tools of protein chemistry and DNA sequencing have been used to unravel the protein origins of these cleaved peptide products. ME was found to be part of a pituitary precursor, β-LPH, which can be enzymatically digested to produce β-endorphin, a potent OP of which ME comprises the amino terminus [79]. This, however, does not appear to be the primary precursor source for the enkephalins. A precursor molecule of M_r of approximately 30 000, termed proenkephalin, has been isolated from human adrenal medullar which contains sequences for one LE, four MEs, and one each of the C-terminal extended ME sequences ME-Arg^6-Gly^7-Leu^8 and ME-Arg^6-Phe^7 [85]. This large protein is processed down to numerous smaller pieces from M_r 500 to 18 000 for further cleaving to obtain enkephalins [86].

Both RIA and RRA have been used to detect enkephalins and the other opioid-like peptides. RRA is less specific so it usually gives higher values and therefore is better suited for identification of new OPs, while specific RIAs coupled with HPLC allow measurement of an individual OP. For example, using both RIA and RRA Simonnet et al. [87] could conclude that there is no direct correlation between ME and general OP levels in cerebrospinal fluid (CSF) in patients with and without vertebral disk disease-associated chronic pain. The use of RIA has also shown that migraine patients have elevated ME levels, while patients with chronic back pain or those receiving non-steroidal anti-inflammatory drugs have decreased ME levels [87]. It has been suggested that patients with elevated OP levels in CSF may have an inhibition mechanism regulating ME release after chronic stimulation of the opiate receptor, the analogy being that heroin addicts also have lower CSF ME levels than control patients [88]. Since circadian rhythms in pain sensitivity was demonstrated in humans [89], it has been shown that ME in plasma and CSF follows this rhythm with low values between 4:00 and 8:00 p.m. [90]. This can present a problem in assessing basal enkephalin levels in the clinic, because immunoreactive ME in human CSF over a 24-h period can vary from 20 to 5000 pg/ml [90]. RIA in fresh mass vasa deferentia indicates that man has 30 .6 $\pm$ 5.6 pmol/g LE and 89.6 $\pm$ 19.3 pmol/g ME [80]. The magnitude and ratio of these values can be quite different in other species. For example, levels in the bull are as high as 207.1 $\pm$ 26.4 pmol/g LE and 393.1 $\pm$ 50.3 pmol/g ME, while the dog has as little as 16.2 $\pm$ 1.2 pmol/g LE and 19.9 $\pm$ 2.0 pmol/g ME [80].

Enkephalins and enkephalin-containing polypeptides are commonly isolated from tissues by acid extraction, GPC, and then RP-HPLC with RIA or RRA detection. Such a procedure was used by Yoshimasa et al. [83] for the isolation of ME, LE, ME-Arg^6-Gly^7-Leu^8, and ME-Arg^6-Phe^7 from human phaeochromocytoma. GPC on Sephadex G-50 with 0.1 *M* acetic acid was followed by RP-HPLC on an Altex Ultrasphere octyldecyl (ODS) column with 10 m*M* ammonium acetate pH 4.2 and methanol. Detection of individual enkephalins required the use of four antisera with low cross-reactivity. Treatment of high-molecular-weight material recovered from GPC columns with trypsin and/or carboxypeptidase-β increased OP immunoreactivity [81, 91–93]. This trick works to free encased immunoreactive peptides because nucleotide sequencing has shown that these OPs are flanked by pairs of dibasic amino acids. Several laboratories have used TFA and propanol [94] or acetonitrile gradients [92, 93] on C_{18} reversed-phase columns for the final purification of enkephalins and endorphin-containing polypeptides or resolution of closely related enkephalin analogues [95]. Some laboratories have also chosen optical detection by post-column derivatization with fluorescamine [96]. This method is more sensitive than UV detection and is compatible with a variety of buffer systems.

In the last few years it has become clear that endogenous opioids have a significant effect on the human periphery and CNS. Enkephalin cosecretion with catecholamines from the adrenal medulla suggests a role in stress response, while differential serum concentrations as a function of pain suggests another role. Correlations have to be made such as which types of pain are associated with which particular enkephalin, endorphin, or other specific OP level in serum and other tissues. These isolated peptides have to be purified and low cross-reactive antisera produced so that RIA or RRA evaluation can be more precise. After these clinical evaluations have been made the therapeutic use of OP agents may be an important modality in the treatment of disease states. Another area of active research has to do with enkephalin stability because endogenous opioids in general are rapidly degraded in the blood [97]. Several researchers are working in the area of protease-resistant, orally active peptide analogues (see ref. 97). HPLC methods to monitor these potential drug analogues in serum have to be very efficient and selective because some may possibly differ only as D-amino acid substitutions.

2.4.7. Growth hormone

Growth hormone (somatotropin or GH) is active in the regulation of a number of growth processes. Thyroid hormone and GH act synergistically during development to achieve normal growth rates. Many actions of GH are mediated through plasma polypeptides known as somatomedins (types A and C are well defined) and insulin-like growth factors I and II. These polypeptides mediate growth by binding to specific cell surface receptors and are dependent upon release of GH. They also stimulate sulfate incorporation in cartilage, produce insulin-like pleiotypic actions, and act as mitogenic stimulants for various cell and tissue types [98]. Other somatomedin-like peptides have been purified and await biological activity assessment [98].

GH is a straight-chain polypeptide hormone of 191 amino acids in length with an M_r of 22 000 and contains two disulfide bridges. It is the mature form of an M_r 28 000 pre-growth hormone produced in acidophilic cells of the anterior pituitary. GH and prolactin are similar in size and structure and share some similar biological properties. The placenta produces proteins which have both GH and prolactin-like activities. They cross-react with antibodies directed against GH, prolactin, or placental lactogen. Bovine GH is biologically inactive in humans but is active in murine and avian species; however, some proteolytic fragments have been found to be active in humans. Primate GH differs from human growth hormone (hGH) in only four residues [99] and is fully active in humans. RIA data in the literature are variable and usually lower than estimates based on RRA or biological assays. This is because RIA is based on immunodetection of a specific structure. When a protein is heterogeneous, the cross-reactivity of related forms can be very low, which is particularly the case for hGH. When extracted from the pituitary gland it is known that hGH is actually a heterogeneous mixture of size and charge forms which give several pools of immunoreactivity on GPC columns. The M_r 22 000 monomeric single-chain hGH described above accounts for only about 85% of immunoreactivity [100]. An M_r 20 000 variant with fifteen deleted amino acid residues accounts for about 7% of immunoreactivity, and 5–10% is composed of N-acetylated, deaminated, and other cleaved structures. Some of these hGH variations that have been defined include one acetylated form, two deaminated forms, and three proteolytic cleaved two-chain forms [100]. The two-chain forms reported have greater biological activity than the single-chain M_r 22 000 monomer [100], while the M_r 20 000 form has full growth-promoting bioactivity in rat even though it has poor GH receptor binding affinity compared to the M_r 22 000 version [101]. Therefore it is important to plan and expect a heterogeneous array of GPC immunoreactive peaks when isolating hGH. Also of note, it has recently been reported that after ^{125}I-labelled hGH is injected in human plasma a minimum of 15% in circulation becomes bound to an M_r 60 000–65 000 plasma component in a 1:2 molar ratio to form an M_r 85 000 complex [102]. This component was not found to be albumin or an immunoglobulin, and is still under investigation.

Iodination of hGH with ^{125}I (half-life 61 days [103]) by the lactoperoxidase technique [104] can be used to develop a double polyclonal antibody RIA for hGH which reacts with all hGH forms [100]. In this method the first RIA has high immunoreactivity with the M_r 22 000, cleaved, and desamide hGH forms, while only about 30% reactivity with the M_r 20 000 form. The second RIA is then developed for high immunoreactivity with the M_r 20 000 hGH. 200-Fold purification with 65–73% recovery by simple immunoextraction can be achieved by passing 10:1 diluted plasma in phosphate-buffered saline (PBS) over an immunoadsorbent column as outlined by Baumann et al. [100]. Ideally, it would be best to have specific RIA kits for the various hGH types. Some progress has been made in this area, for example a specific monoclonal anti-hGH antibody has been used to discriminate between mono- and poly-hGH immunoreactivity [105]. Immunoadsorbent techniques prior to RIA have been recommended for assaying basal level hGH to increase sensitivity by about 50-fold to 25 pg/ml [106, 107].

This is especially important when trying to quantitate basal levels because they can be less than 1 ng/ml of plasma. Having developed sensitive RIA methods it is now known that hGH secretion bursts occur nocturnally over 1–2 h periods, usually in the first half of deep sleep. Fasting adult serum levels are >2 ng/ml except during burst periods [108], where the differential between plasma basal and stimulated levels can span orders of magnitude in the range 34–60 000 pg/ml [106]. Women are known to have higher hGH levels at both basal and burst periods as compared to men [106]; this could be due to the effect estrogen has on hGH secretion. The male hGH level can also be increased by estrogen administration [109]. Using RIA methods the highest hGH concentrations are found in the hypothalamus but also found in other organs or systems such as the pancreas or CNS. The half-life in circulation is about 20–25 min [108], but the metabolic clearance of hGH is independent of plasma concentration because of the fluctuating levels caused by episodic pituitary secretion [106, 110, 111].

Isolation of hGH with RIA monitoring has been done by a variety of methods which usually includes an initial salt precipitation or immunoaffinity step, followed by GPC to separate monomeric from polymeric forms, then finally HPLC. For example Chang et al. [112] have purified hGH from acromegalic pituitary tumors by 20–40% ammonium sulfate fractionation followed by Sephadex G-100 GPC, and finally RP-HPLC consisting of 10-μm silica bonded with C_{18}, using a linear acetonitrile gradient with 0.1% TFA. Elution of hGH occurs at just under 50% acetonitrile, indicating that hGH is relatively surface-hydrophobic compared to many other hormone proteins. Detailed analysis comparing hGH to tumor-derived hGH [by polyacrylamide gel electrophoresis (PAGE), sodium dodecyl sulfate PAGE (SDS-PAGE), HPLC, peptide mapping, amino acid composition, and N-terminal sequence analysis] has been done by Chang et al. [112] which makes a good starting reference article for characterizing hGH. Extensive gel analysis of various hGH forms have been outlined by Baumann et al. [113] and are further discussed in Section 3.3. Another procedure illustrating the use of RP-HPLC as the final purification step in the isolation of GH from monkey pituitary glands used a Vydac 201TP104 column (C_{18}), 0.1% TFA, and a linear isopropanol gradient from 35 to 45% over 30 min [99]. This article and another by Kohr et al. [114] serve as nice references for GH tryptic analysis. Patience and Rees [115] studied reversed-phase (Altex Ultrapore RP5C) and anion-exchange (Toya Soda TSK DEAE 5-PW) HPLC methods for separation of hGHs from freeze-dried and fresh pituitary extracts. M_r 20 000, M_r 22 000, dimer, reduced, and deaminated standards were also monitored. This paper includes a convenient table listing the selectivity differences between RP-HPLC and anion-exchange HPLC for these various hGHs and comparative RIA recoveries (78–85% for reversed-phase versus 84–93% for anion-exchange chromatography). It was noted that if vacuum desiccated as opposed to freshly eluted fractions were assayed, recoveries dropped substantially to 36 and 74% for reversed-phase and anion-exchange chromatography, respectively. This is an important point to consider when establishing an assay protocol. To prevent hormone adsorption to plastic collection tubes, these authors take the precaution of adding bovine serum albumin (BSA) or Triton X-100 to tubes before collecting fractions.

There have been several instances where non-HPLC steps have shown to be very effective. One case in particular was demonstrated in a study by Baumann et al. [100], where they were able to isolate and identify several isoforms of circulating hGH by the use of a simple immunoaffinity column. The variants were then separated by anion-exchange chromatography on DEAE-Sephadex. It is plausible to assume that the anion-exchange step could be adapted to anion-exchange HPLC to speed up the process and possibly increase resolution.

Historically, hGH was first extracted from acetone-preserved pituitaries for therapeutic use, but the advent of purified recombinant hGH [114] has made this material readily available for the treatment of growth abnormalities. Monitoring hGH levels in the treatment of dwarfism and acromegaly are important clinical assays, and the specific importance or significance of the various hGH forms found in plasma has yet to be elucidated.

2.4.8. Insulin

Insulin is a highly conserved 51-amino acid residue protein hormone which has been isolated in more than 25 vertebrate species [116]. In all species, 19 of 51 positions are conserved, while all mammalian structures differ by no more than 8 positional changes [117]. This hormone and its receptor have served as a model for virtually all basic research on protein hormone–receptor interactions and is the central theme in the study of diabetes. Proinsulin is cleaved in the β-granules of the islets of Langerhans in the pancreas to form the mature insulin and C-peptide, which are secreted into circulation in equal amounts. The clinical measurement of C-peptide does not cross-react with insulin antibodies in serum, so it gives a good measurement of insulin secretion. Levels greater than 1.5 g/l C-peptide confirms hyperinsulism, whereas low C-peptide with high insulin serum levels indicates factitious hypoglycemia [108]. Normal insulin serum values are 4–30 μU/ml (mean 17 μU/ml), obese subjects have higher levels. Measurement of insulin by RIA is done in serum because plasma measurements are affected by the presence of heparin. Plasma measurements require the addition of EDTA and the use of double antibody systems. Following glucose ingestion it takes about 30–60 min to reach maximal insulin serum level in the human. Biologically, it is known that insulin binds and phosphorylates the β-subunit of its receptor, then is internalized by endocytosis in insulin-sensitive tissues, and finally processed to low-molecular-mass TCA-soluble materials [118, 119]. The significance of each of these steps is still under investigation by several groups. This review will highlight the separation and isolation of radiolabelled insulins, various species-specific insulins, and highlight how the HPLC identification of new mutant human insulins impacts diabetes research.

Radiolabelled insulin for receptor binding studies is commonly done by the chloramine T method of iodination, followed by solid-phase extraction [120], and finally HPLC [121, 122] for final purification. Gliemann et al. [123] showed that of the four tyrosines in insulin, the iodination of Tyr14 on the A chain ([A_{14}-^{125}I]insulin) gave the most similar bioactivity with respect to native insulin. RP-HPLC procedures for ^{125}I-labelling and purifying [A_{14}-^{125}I]insulin have been published [120, 122] and have quickly supplanted PAGE identification meth-

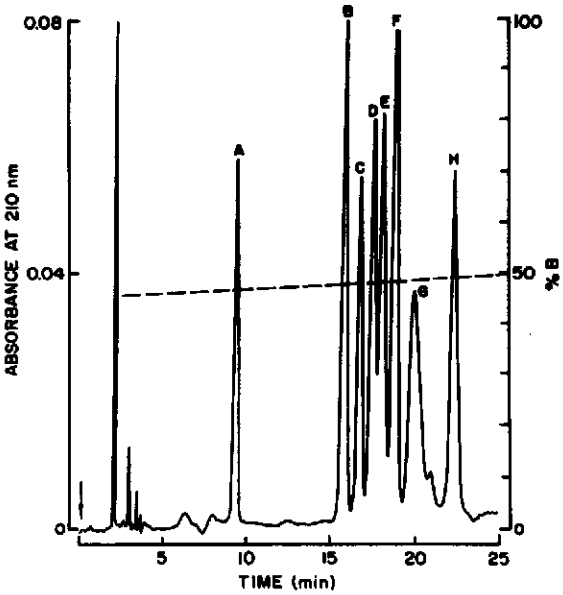

Fig. 5. Separation of insulins from (A) chicken, (B) bovine, (C) ovine, (D) rabbit, (E) human, (F) porcine, (G) rat type I, and (H) rat type II. Column, V-1830-4, Vydac C_4; load, 5 μg each; flow-rate, 2 ml/min; pressure, 2900 p.s.i. (ca. 20 MPa); solvent A, 0.1% (v/v) TFA; solvent B, 0.1% (v/v) TFA in acetonitrile (60:40); gradient: from 45% to 50% B in 25 min; detection, 0.08 a.u.f.s. at 210 nm. Reprinted from ref. 126 with permission.

odology. The procedure of Stentz et al. [120] allows for about 80% specific labelling of the A_{14} residue, with the remainder mostly on the A_{19} tyrosine. After solid-phase extraction and DEAE cellulose elution, [A_{14}-^{125}I]insulin was easily separated from [A_{19}-^{125}I]insulin by HPLC on two μBondapak columns in tandem with TFA as the IPA and acetonitrile in the mobile phase. Pingoud and Trautschold [122] achieved similar results using a Zorbax-TMS column with phosphate buffer and acetonitrile. To minimize diiodotyrosine derivative and favor A_{14}-^{125}I over A_{19}-^{125}I, it is recommended that the process be done at a constant iodine-to-peptide ratio of 1:10 between pH 5 and 8 [122].

The application of HPLC to insulin is so widespread that the hormone is often used as a chromatographic standard. Classically, insulin was purified from pancreas by GPC and cation-exchange methods. RP-HPLC has been substituted for cation-exchange chromatography because it is quicker [124] and eliminates the problem of using salts. Several high-resolution RP-HPLC methods for insulin isolation are described in the literature [117, 125–132]. Separations can be achieved on either small-pore [125] or large-pore [126] supports. While IPAs usually used for insulin separation include 0.1% TFA, 0.1% HFBA, and phosphate, reports of TEAP [126] or TEAP plus sodium perchlorate [131, 132] indicate excellent selectivity. Isolation of individual chains of recombinant insulin have been done with reduced levels of TFA (0.0125%) or 0.5% formic acid [128]. Fig. 5 demonstrates the separation of eight closely related mammalian insulins. This is impressive when the evolutionary conservation of insulin is examined.

Rabbit and human insulins can be separated even though differing by only a methyl group (threonine is substituted for serine at residue 30 on the B chain). This same study by Rivier and McClintock [126] demonstrated the utility of three different (C_{18}, phenyl, and C_4) reversed-phase bonded phases for the separation of closely related insulins. IPAs can also greatly affect peptide retention times. These researchers [126] found that TEAP at pH 2.25 allowed greater selectivity and faster elution than TFA, although some insulin peaks eluted with broader peak widths. TFA was found to be better for the later-eluting (more surface-hydrophobic) insulins [126] as shown for the isocratic elution of five human and four porcine insulins in Fig. 6a, which employs a C_{18} column, 29.7% acetonitrile, and 0.10 *M* phosphoric acid–0.02 *M* triethylamine–0.05 *M* sodium perchlorate at pH 3 [131–133].

The peaks in Fig. 6b–d illustrate the powerful combination of HPLC separation and RIA detection for the identification of three different insulin variants isolated from sera of three unrelated hyperglycemic and hyperinsulinemic patients that respond normally to insulin. Reduced retention times suggested that the mutant insulins in Figs. 6b and 6c were more hydrophilic. This turned out to be the case as the mutation in Fig. 6b was eventually established as a serine substitution for phenylalanine in position 24 of the B chain (defined as insulin Los Angeles). The mutant insulin in Fig. 6c had a leucine substituted for phenylalanine in position 25 of the B chain (insulin Chicago). In the case of Fig. 6d, the increased retention time of the mutant suggested an exchange for a more hydrophobic residue [132]. This was confirmed a few years later after the patient's leucocyte DNA had been harvested, the insulin gene cloned and sequenced, and corresponding amino acid sequence deduced. The mutant in Fig. 6d was determined to have a leucine substituted for valine in position 3 of the A chain [134] (insulin Wakayama). Additional proof of identity consisted of solid-phase peptide synthesis of these mutant insulins and noting that RP-HPLC of these synthetic insulins co-eluted with isolated variants.

The ability to develop highly selective HPLC systems for insulin separations has also advanced synthetic structure–function studies. For example as a result of selective HPLC purification of synthetic insulin analogues, it was made possible to determine that replacements in position 25 of the B chain disrupt important side-chain contact between insulin and its receptor. In addition, changes in position 24 of the B chain were found to cause conformation changes in the hormone [133]. The incidence of insulin gene mutation in man and its relation to the diabetic state is still being defined and relies heavy upon new HPLC immunoreactive isolates from sera. Such studies are also being done for insulin autoimmune syndrome. In this disease state, hyperinsulinemia and hypoglycemia are accompanied with autoantibodies to insulin in patients who have never been injected with insulin [135, 136]. RP-HPLC elution revealed that mutant insulins in this case are more hydrophobic than normal human insulin [130, 137] but their structures have yet to be determined.

2.4.9. β-Lipotropin

Human β-lipotropin (β-LPH) is an 89-amino acid residue protein hormone [138] with strong lipolytic and glucose metabolism activities [139, 140]. β-LPH

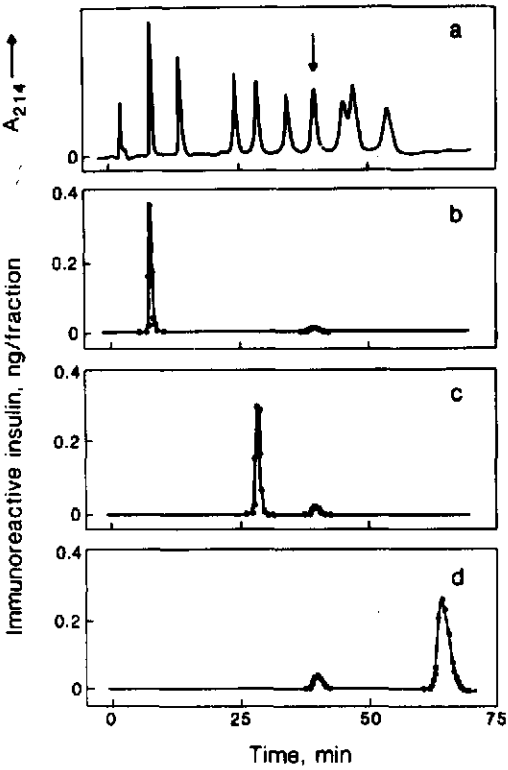

Fig. 6. RP-HPLC separations of abnormal insulins isolated from the serum of human subjects. (a) A mixture of insulin standards visualized at 214 nm; the peaks correspond, from left to right, void volume peak, human [Ser^{B24}]insulin, human [Ser^{B25}]insulin, porcine [Ala^{B24}]insulin, human [Leu^{B25}]insulin, porcine [Leu^{B25}]insulin, normal human insulin (arrow), human [Leu^{B24}]insulin, normal porcine insulin, and porcine [Leu^{B24}]insulin. (b–d) Immunoaffinity-purified insulin isolated from the serum of three unrelated human subjects who secrete abnormal insulins. Insulin content was determined in all fractions by RIA, but data points are only shown for those fractions where immunoreactive insulin content was above the limit of detection. Note that a small peak of normal insulin appears in each of panels b, c, and d. (b) Mutant identified as human [Ser^{B24}]insulin [115]; (c) mutant identified as human [Leu^{B25}]insulin [115]; (d) mutant identified as human [Leu^{A3}]insulin [115]. Mobile phase, isocratic, 29.7% acetonitrile in 0.10 *M* phosphoric acid–0.02 *M* triethylamine–0.05 *M* sodium perchlorate (adjusted to pH 3.0 with sodium hydroxide); column, C_{18} Altex Ultrapore 5 μm, 25 cm × 0.46 cm); flow-rate 1 ml/min at 22 °C; detection by absorbance or RIA [137]. Reprinted from ref. 133 with permission.

and ACTH are derived from pro-opiomelanocortin (PMOC) [141], and β-LPH is further processed to various opiate endorphin peptides. The mean plasma β-LPH level in normal subjects is about 4–5 fmol/ml [142, 143]. β-LPH plasma levels are increased in response to serotoninergic agonists [144] and physical stress [145–147], which may be linked to pituitary cyclic AMP regulation [148]. During labor β-LPH levels rise to almost 70 fmol/ml [143]. Molar ratios of plasma β-LPH and β-endorphin are lower in patients with Addisons disease and higher in patients with chronic bone pain [142].

A common method for bioassay is induced lipolysis in isolated rat cells, but a

drawback of this method is that it is not very specific for β-LPH. The most efficient method for monitoring β-LPH during purification or in the clinical laboratory is by RIA [142, 149, 150]. Since β-LPH contains the sequence of β-endorphin (residues 58–89 of β-LPH) most antisera raised against β-endorphin will also cross-react with β-LPH. For this reason β-LPH is usually separated from β-endorphin on a GPC column prior to assessing cross-reactivity. Although recently it has been reported that the antigenic determinant for one β-LPH-specific RIA is the N-terminal 1-36 region of β-LPH [149]. A faster method uses cation exchange [150, 151] on a Pharmacia MonoS™ HR 5/5 column, with an ammonium acetate gradient in the presence of 20% acetonitrile, to separate β-LPH from β-endorphin in only 15 min [150]. Adding [^{125}I]β-endorphin internal standard to the sample before HPLC injection makes peak identification relatively easy.

Several other HPLC methods are available to use for the isolation and RIA quantitation of β-LPH. Human and porcine (91 amino acid residues) β-LPH can be purified from pituitary glands by carboxymethyl cellulose chromatography [152, 153] followed by RP-HPLC. Various methods include a Kontron C_{18} column with methanol gradient and phosphate at pH 2.43 [154], a Hypersil-ODS column with acetonitrile gradient and phosphate at pH 2.1 [155], and a μBondapak CN column with acetonitrile gradient and TEAP at pH 3.0 [156]. Reversed-phase thin-layer chromatography (RP-TLC) has also been used for the separation of β-LPH, β-endorphin, and enkephalins. Whatman C_{18} RP-TLC plates were developed with propanol–0.1 *M* phosphate buffer pH 4.1 (30:70) [157]. Richter and Schwandt [158] conducted a study to evaluate various HPLC supports for the separation of neuropeptides. In particular they compared C_{18} LiChrosorb, Hamilton PNP-1, and Aquapore RP300, and found superior resolution on the Aquapore column for a collection of 27 regulatory peptides and proteins using 0.1 *M* phosphate at pH 2.1 and a linear acetonitrile gradient (Fig. 7). Collected fractions were easily desalted by SEC on a TSK 2000 P SW column to remove the phosphate. Alternatively, volatile TFA or HFBA can be substituted for phosphate.

2.4.10. Parathyroid hormone

Parathyroid hormone (PTH) is a single-chain polypeptide containing 84 amino acids with an M_r of 9500. PTH assay is used in the diagnosis of primary hyperparathyroidism [159]. Like most peptide hormones, PTH is a cleavage product of its prohormone. It is during this cleavage that PTH is transferred from endoplasmic reticulum to the Golgi apparatus and finally into the secretory vesicle where it is stored in parathyroid cells. PTH is one of three substances (PTH,CT, and calciferol) which regulate plasma calcium levels at about 10 mg/dl. Low plasma calcium levels release PTH directly into circulation from the secretory vessels. PTH has a short biologic half-life in which it is cleaved into a major inactive carboxy fragment and a smaller amino fragment with partial activity. This event leads to variable results when comparing RIA data to biological activity because anti-PTH antibodies recognize active and inactive PTH fragments as well as intact PTH. Bioassay for PTH may be done by assessing adenylate

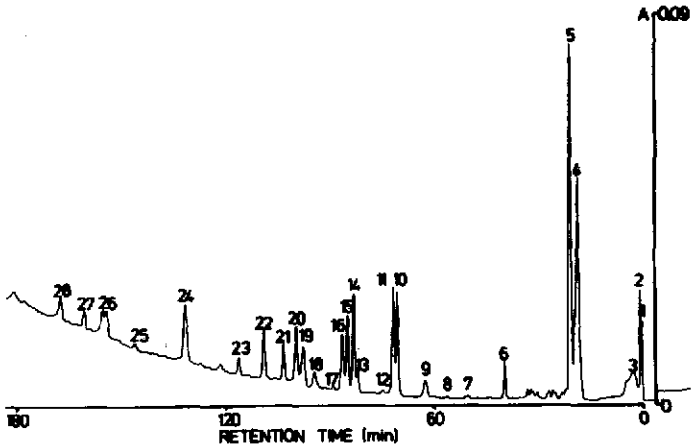

Fig. 7. Separation of 27 peptides and proteins on a 25 cm × 0.4 cm Aquapore RP300 column. Of each peptide or protein, 500–1500 ng were dissolved in buffer A and injected onto the column in a 100-μl volume. Buffer A, 0.1 *M* sodium dihydrogen phosphate adjusted to pH 2.1 with orthophosphoric acid; buffer B, methanol; gradient, 10 to 65% B in 180 min at 1 ml/min at room temperature. A = Absorbance at 225 mm. Peaks: 1 = prolactin; 2 = hGH; 3 = LH; 4 = FSH; 5 = TSH; 6 = TRH; 7 = β-LPH$_{88-91}$; 8 = ACTH$_{1-4}$; 9 = β-LPH$_{1-10}$; 10 = β-LPH$_{39-45}$; 11 = ACTH$_{4-11}$; 12 = β-LPH$_{61-65}$; 13 = LRF contamination; 14 = β-LPH$_{62-91}$; 15 = LRF; 16 = β-LPH$_{66-91}$; 17 = LE; 18 = β-MSH, porcine; 19 = ACTH$_{4-10}$; 20 = ACTH$_{1-24}$; 21 = β-LPH$_{61-76}$; 22 = ACTH$_{1-13}$; 23 = γ-MSH; 24 = ACTH$_{18-39}$; 25 = ACTH, porcine; 26 = β-endorphin, human; 27 = β-LPH$_{66-89}$; 28 = β-LPH, human. Reprinted from ref. 158 with permission. Abbreviations: LH = luteinizing hormone; FSH = follicle-stimulating hormone; TSH = thyroid-stimulating hormone; TRH = thyrotropin-relasing hormone; LRF = luteinizing hormone-releasing factor.

cyclase synthesis rates in cloned rat osteosarcoma cells [160] or by stimulation of glucose-6-phosphate dehydrogenase activity in cultured renal cells [161]. In the clinical setting RIA is the preferred method of assay with normal PTH values in serum ranging from about 0.5 ng/ml to undetectable levels [162], but cleavage as noted above can lead to variable results.

PTH is cleared from plasma primarily by the liver which is the site of proteolysis for C-terminal PTH fragment formation [163]. Several different carboxy fragments with a single N-terminal fragment have been isolated [164]. Measurements of immunoreactive PTH can be used to discriminate non-parathyroid from parathyroid induced hypercalcemia [165]. Plasma extract immunoreactive PTH peptide profiles for osteoporotic, hyperparathyroid, and pseudohyperparathyroid patients have been characterized [166]. RIA specific for intact PTH has been preferred to C-terminal PTH for monitoring graft function in patients receiving parathyroid tissue autografts [167]. It is well known that C-terminal PTH immunoreactivity yields more useful information than N-terminal reactivity in the clinical evolution of parathyroid function [162].

Several investigators have employed HPLC for the isolation and purification of PTH [158, 164, 166–173]. Rabbani et al. [168] have published data on an entire series of peptides extracted from human squamous cell and Leydig cell carcinomas which have PTH-like activity. Their purification scheme involved an initial acid extraction followed by tandem chromatography on Bio-Rad Bio-Sil TSK CM-3SW cation- and Bio-Sil TSK DEAE-3W anion-exchange HPLC col-

umns in series. This allows for quick separation of peptides and proteins into basic, acidic, and neutral groups. Each group was then eluted from their respective columns (except for the neutral peptides which passed through both columns) and further separated on a Waters C_{18} μBondapak RPLC column. Zanelli and co-workers [169, 170] employed RP-HPLC to purify PTH from a crude extract of bovine parathyroid gland in a single run. They employed a Hypersil ODS analytical column with 0.155 *M* sodium chloride and eluted with a linear acetonitrile gradient. The result was a clean, well resolved PTH peak from the bovine mixture as shown in Fig. 8. The only drawback with this procedure is that the final preparation is not salt-free, but this can be corrected by buffer exchange with 0.1% TFA in acetonitrile using either a Sep-Pak-type solid-phase extraction procedure or a quick reversed-phase gradient on an HPLC column. Pines et al. [171] purified avian PTH from one-day-old chicks by acid extraction, GPC on Sephadex G-75, and finally RP-HPLC using a C_{18} column with 0.14 *M* ammonium acetate and a linear acetonitrile gradient. Zull and Chuang [172] used RP-HPLC to clean up and separate the radioactive monoiodotyrosine from diiodotyrosine derivative of PTH. Bioactive-labelled PTH was purified by simple isocratic elution on a Waters C_{18} μBondapak with 0.1% TFA in 27% acetonitrile.

In general, RP-HPLC is the mode of choice for purifying protein hormones, provided the elution conditions do not render the macromolecule inactive. After optimizing as best as possible an RP-HPLC fraction, additional resolution may be achieved by ion-exchange HPLC. For example, in the case of synthetic human PTH it was separated from an [Asp^{76}]hPTH analogue (Asp substituted for Asn at residue 76) by cation-exchange HPLC using a Toyo Soda TSK CM-25W column with a sodium chloride gradient in the presence of 20 m*M* phosphate and 10% acetonitrile [173]. Since the single amino acid substitution changed the charge of the hormone, ion-exchange HPLC was a rational approach to take as opposed to RP-HPLC for fine resolution of these closely related macromolecular structures.

3. ELECTROPHORESIS OF PROTEIN HORMONES

3.1. Sample handling

Samples for electrophoretic analysis can be prepared analogous to those for HPLC (Section 2.1.) or, alternatively, they can be prepared by centrifuging body fluids at 200 000 *g* prior to adding the appropriate sample solubilization buffer. Cells and tissue samples need only be homogenized with mortar and pestle and proper protease inhibitors added prior to centrifugation.

3.2. Detection

The most common visual methods for protein detection in the clinical laboratory are Coomassie Blue [174, 175] and a variety of more sensitive silver stain methods [176–180]. In the case of low nanogram amounts or incomplete sepa-

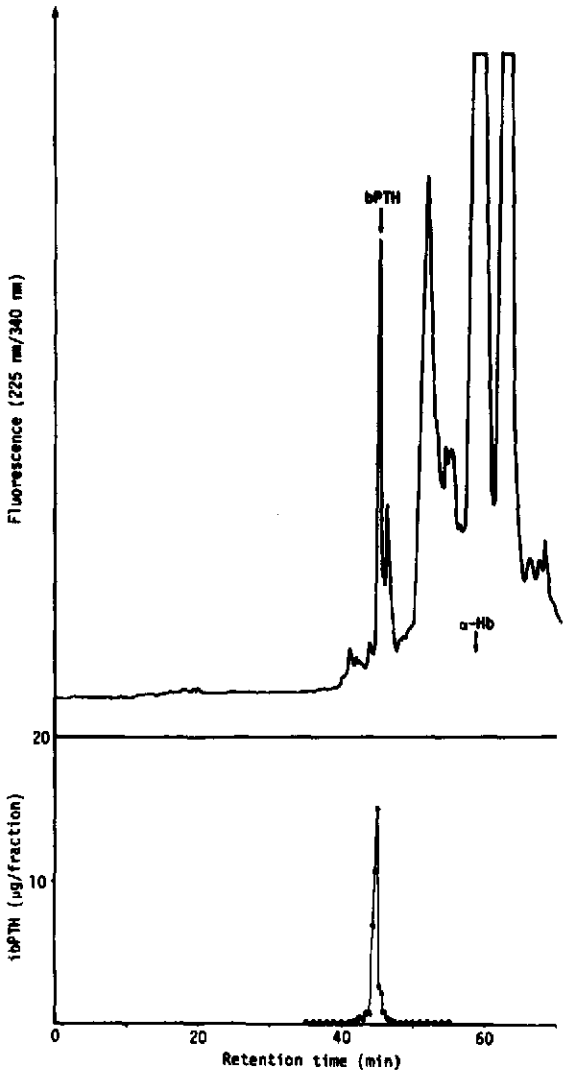

Fig. 8. Gradient elution HPLC of 1 mg crude TCA-precipitated bovine PTH. Column, 150 mm×4.6 mm ODS-Hypersil; flow-rate, 1 ml/min; temperature, 35°C; gradient, 10 to 50% acetonitrile in the presence of 0.155 *M* sodium chloride (pH 2.1) in 60 min. Immunoreactivity (ib PTH) in 18-s eluent fractions are shown below the chromatogram. Elution times of highly purified bovine PTH (bPTH) and hemoglobin α-chain (α-Hb) are indicated. Reprinted from ref. 169 with permission.

ration from other components, gel slices can be cut out and subjected to immunoassay detection.

3.3. Electrophoretic analysis

Electrophoretic analysis of protein hormones is more time-consuming than HPLC, but it is important to recognize that this technique complements HPLC analysis in the research laboratory. Whereas HPLC highly resolves proteins and

peptides by surface charge difference (ion-exchange HPLC) or relative surface hydrophobicity (RP-HPLC), size separation of small protein hormones by SDS-PAGE is superior to size-exclusion HPLC methods. In addition, since IEF separation is based on total net charge of proteins, and not surface charge as is the case for ion-exchange HPLC [181], this technique is a useful alternative for achieving unique resolutions. Coupling IEF and SDS-PAGE for a 2-D gel electrophoretic analysis gives a powerful alternative to HPLC-based separations. The method has been used to resolve complex mixtures of proteins from various body fluids and tissues including plasma, serum, urine, saliva, brain, muscle, and kidney. This sensitive technique allows the investigator to isolate and examine one or several protein spots on a gel with hundreds of other proteins present. For this reason 2-D gel electrophoresis can be used to detect protein abnormalities and may be applied to routine clinical analysis and diagnosis. Tracey and Young [182] and Tracy et al. [183] have presented thorough discussions of general clinical applications. We shall focus on the utility of and difficulties associated with gel electrophoresis of polypeptides, and present a few examples which may be useful in the clinical laboratory.

Electrophoresis of peptides is usually performed using one of two general methods. The first makes use of gels which have acrylamide concentrations greater than 15% and are highly cross-linked. In the second method, urea [184] or glycerol [185] is added to the acrylamide matrix to facilitate sieving of small peptides (see ref. 186 for further discussion). Modifications for fine tuning of these two techniques include gel buffer composition changes, altering running buffers, substitution of various cross-linking agents, and the use of a gradient as opposed to homogeneous polyacrylamide gel content.

Several examples of protein hormones addressed in this review have been size-separated by electrophoresis, including bovine PTH [169], ^{125}I-labelled PTH [172], [^{125}I]insulin [187–189], and human growth hormone [113]. Scintillation counting, RIA determination, and/or Coomassie Blue staining of gel slices are commonly used for detection depending on the nature of the protein hormone. The size separation of hGH species by Baumann et al. [113] with subsequent immunoreactivity determination of gel slices is a good example of the high resolution potential of electrophoresis and its capability to show heterogeneity of patient profiles (Fig. 9). Native PAGE and SDS-PAGE patterns of plasma hGH from various patients in Fig. 9 elucidate size differences that would have been difficult to assess using SEC. Fig. 10 illustrates the IEF charge-based resolution of the M_r 22 000 principle form of hGH from the M_r 20 000 variant and other unidentified acetylated, deaminated, and proteolytic forms.

Many highly refined electrophoretic techniques have recently been reported which enable the investigator to analyze protein hormones over a broad M_r range [186, 190, 191]. Coupling these methods with the more standardized first-dimension charge-based IEF separation gives highly resolved sample profiles.

2-D gel electrophoresis can be applied to the analysis of complex tissue and fluid extractions where column purification of desired peptides is prohibitive or impossible, and ideally for locating new proteins that are not identifiable by standard assay techniques such as RIA and enzyme-linked immunoabsorbant assay.

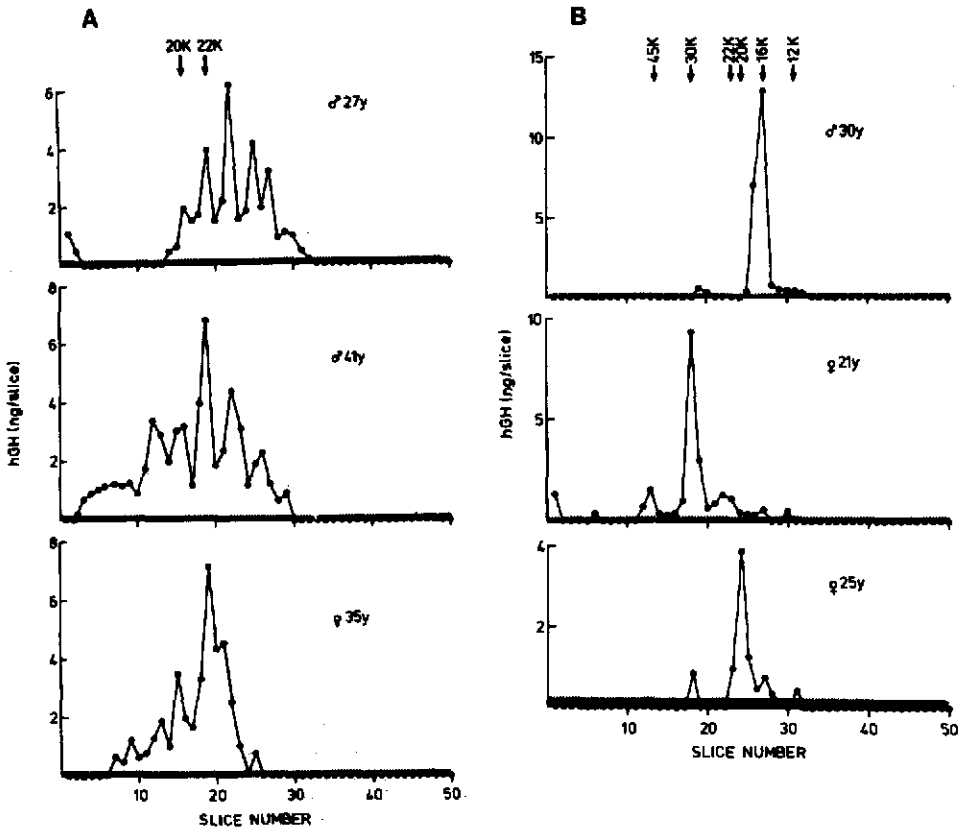

Fig. 9. Patterns of plasma hGH immunoreactivity during basal periods of six different patients. (A) Native PAGE, pH 7.5, 12% acrylamide; (B) non-reducing SDS-PAGE, pH 10, 14% acrylamide. Sex and age of subjects are indicated in figure. Reprinted from ref. 113 with permission.

For example, several laboratories have used 2-D gel analysis to study disease-state protein profiles and compared these to controls [192–196]. The observed differences in protein patterns include existence of aberrant proteins, non-existence or quantitative differences of proteins, and molecular mass or isoelectric point shift forms. An interesting application of electrophoresis is the diagnosis of genetically induced disease states from changes in marker proteins. Sammons et al. [180] found that specific marker proteins appeared or disappeared in diabetic or non-diabetic hamster red cell lysate profiles. This same paper details the use of 2-D electrophoresis for monitoring protein changes in human hepatoma cells upon drug treatment. 2-D gel electrophoresis may possibly be used in the future to monitor decomposition of therapeutic protein hormones in biological samples. We wish to emphasize the capability of 2-D gel electrophoresis for resolving minor peptide differences within a complex biological extract. Fig. 11 shows a 2-D separation of mouse pituitary extract. The low-molecular-mass array of silver-stained spots at about 2.5-6 kD are isoform variations (charge variants) of the peptide hormone ACTH. These variations visually explain the broad ACTH

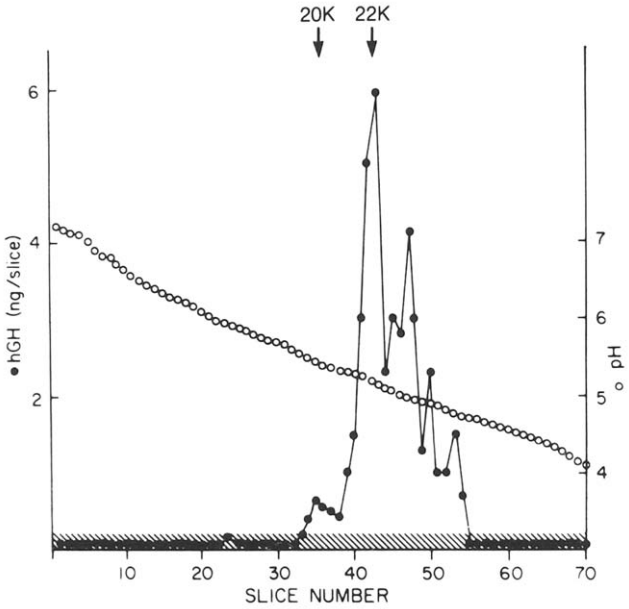

Fig. 10. IEF pattern of hGH pituitary extract standard after immunoadsorbent chromatography purification. Molecular mass markers locating the major form of hGH (M_r 22 000, 22K) and single-chain variant (M_r 20 000, 20K) are shown. Reprinted from ref. 100 with permission.

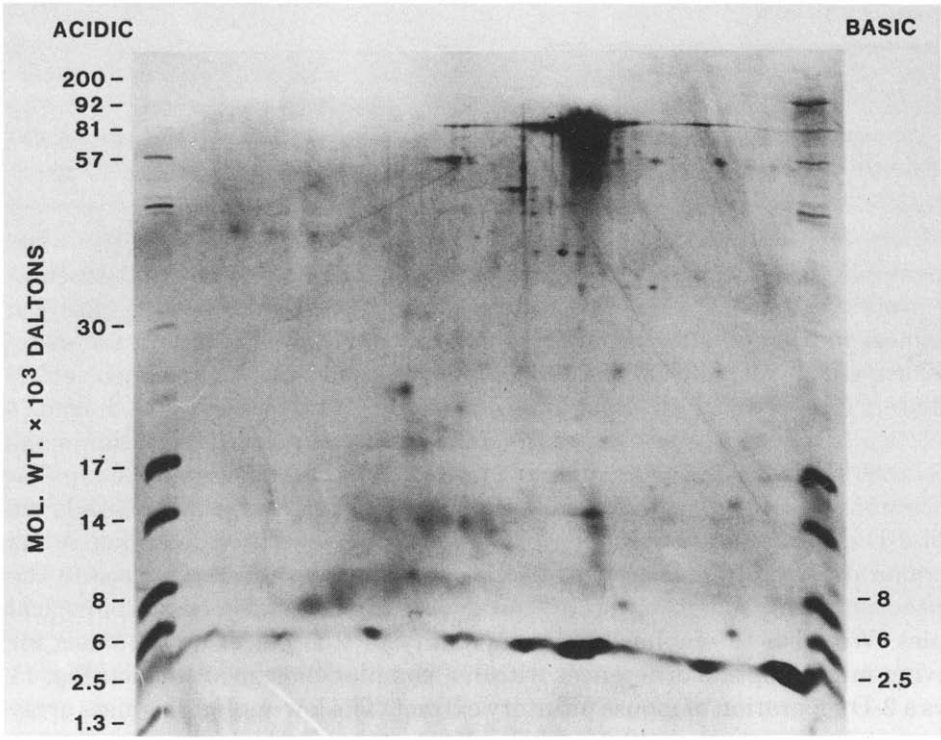

Fig. 11. 2-D gel electrophoretic separation of low-molecular-weight mouse pituitary polypeptides. Normal (C57) mouse pituitaries were acid-extracted by the method of Bennett et al. [197] with addition of 0.001% PMSF in extraction medium. 2-D electrophoresis was performed as previously described by DeWald et al. [186]. The prominent array of spots migrating at 2.5–6 kD along the basic end well into the acidic region corresponds to [illegible] of ACTH

immunoreactivity peaks often seen for HPLC profiles of biologic samples. 2-D gel analysis can also help identify new protein hormone variants that do not have sufficient RIA cross-reactivity for identification. Finally, direct protein sequencing of 2-D gel spots by electroblotting [198, 199] or high-pressure gel elution [200] procedures have recently allowed researchers to obtain structural information directly from gels without the need of HPLC-based separations.

General use of electrophoresis in the clinical laboratory necessitates that this technique be automated, routine and standardized. It will be essential to adopt one of several very similar general methods in order to decrease the variability from laboratory to laboratory. The ISO-DALT [201, 202] technique is fairly routine and allows the researcher to compare protein maps with those already in existence. Although 2-D gel electrophoresis is relatively time-consuming, expensive, and requires specialized equipment, it is available for expanding the field of assay possibilities in the clinical laboratory.

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5. SUMMARY

The purpose of this review is to highlight modern techniques in HPLC and electrophoresis used for protein hormone separations. The advent of biotechnological methods for production of synthetic polypeptides and recombinant proteins will have a significant future impact on the types of therapeutics and metabolites that need to be monitored in the clinical laboratory. The protein hormone examples given in this work were selected because of the comprehensive body of separation science literature and not necessarily for their future importance in medicine. The intention was to present an array of general methods and techniques which may be useful to the clinical investigator for analysis of any protein hormone.

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