High-Performance Liquid Chromatography of Peptides and Proteins. Pharmaceutical and Bio-medical Applications

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Abstract
The power of the technique of high-performance liquid chromatography as an analytical tool is becoming widely recognized. In the field of peptide synthesis the method is being employed for both quality control and preparative isolation. Similarly, naturally occuring polypeptide hormones are being isolated and characterized employing the method. Its use in identifying amino acid residues in proteins which were post translationally modified with pharmaceutical agents has been illustrated. Procedures, as employed in our laboratories, are given and applications illustrated for various problems which have arisen related to polypeptides, or chemical reactions thereon, which are of bio-medical/pharmaceutical relevance.

In any endeavor to synthesize or isolate a compound, which for purposes of this article is a peptide or small protein, it is necessary to have or develop the means of controlling the homogeneity of the final products. In some cases it might also be possible to use exactly the same analytical technique for the isolation itself provided the method can be "scaled-up" to accomodate the larger quantities. The appropriate technique should therefore offer high sensitivity, resolution and yields while maintaining low operating costs. As a general method it should also be relatively easy to carry out, fast and, when possible, amenable to total automation. During the past few years the improvements made on commercially available high-performance liquid chromatographic (HPLC) equipment has yielded a product which closely fulfills the aforementioned prerequisites.

It is now possible to carry out analytical and preparative isolations of large peptides or proteins and peptides arising from chemical or enzymatic cleavages via HPLC. Their characterization can then be carried out by amino acid analysis on HPLC-based instruments and the determination of their primary sequences is dependent on HPLC identification of Edman-type degradation products. The technique has also found wide application in the analytical laboratory as a means of determining not only the degree of homogeneity but also in the identification of specific substances in such widely diverse fields as clinical chemistry and forensic medicine.

The aim of this communication is to present some of our observations concerning the HPLC of peptides and small proteins, from both natural and synthetic sources, which have applications in the areas of medicine and pharmacy. Its use as a means to identify and further characterize *in vivo* and *in vitro* modifications (post translational) are also presented. The techniques as used in our laboratories are given and specific examples of some HPLC applications presented. For the more technical details on specific methods, as well as literature pertaining to the work of other groups, see the references given at the conclusion of this article.

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Techniques

Instruments

The basic two-pump HPLC unit for gradient elution is illustrated in Fig. 1. In this arrangement pumps A and B (Altex model 110) are controlled via a microprocessor (Altex model 420 or Kontron model 200) to deliver solvents A and B to the mixing chamber (Altex or self-construction). Gradient formation is thus controlled through the percentage of buffer delivered by each pump

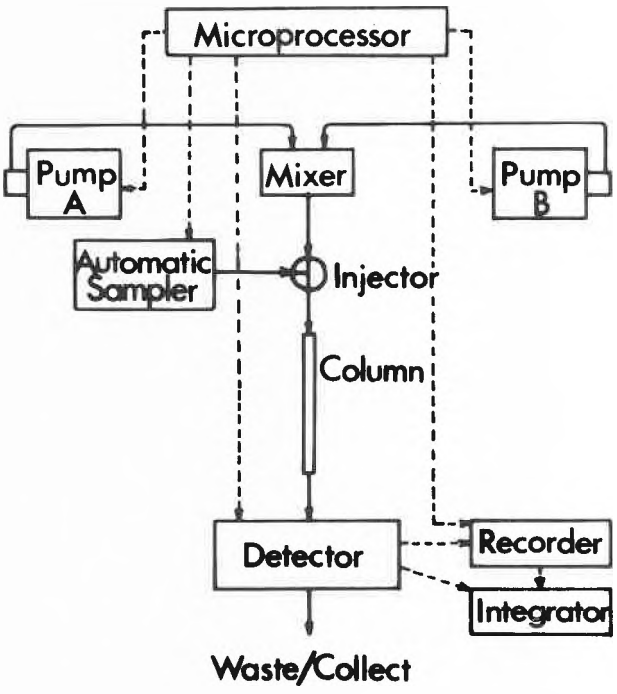

Fig. 1: The basic HPLC instrument.

and mixing occurs at the back-pressure developed by the flow rate, temperature and column packing being used. The other most commonly employed arrangement utilizes a single pump to deliver the gradient which is formed (at atmospheric pressure) by a series of microprocessor-controlled proportioning valves. Located prior to the column is either a manually operated (Rheodyne model 7125) and/or an automatic injector (Kontron Model 100) for sample application via sample loops ranging in volume from 10–500 μl.

The detection system utilized is dependent upon the absorbance properties of the buffers (e. g. their compositions) which, in turn, are determined by the sample being chromatographed. In those cases where neither peptide solubility nor aggregation are factors, buffers which allow detection at $\leq$ 220 nm are used. Conversely, pyridine-based buffers of high ionic strengths which improve peptide/protein solubility require a secondary means of detection, usually fluorescence. Measurements in the UV are conveniently performed using spectrophotometers equipped with micro flow-through cells (Uvikon 725, 8 μl cell). Similarly, fluorescence detection of the reaction products of a fluorogenic reagent (fluorescamine or o-phtalaldehyde) with primary amino groups of the peptide/protein are carried out with a fluorometer (Spectra/Glo Fluorometer, 65 μl cell; Amino Fluoro-Monitor, 70 μl cell). Although not indicated in Fig. 1a means of maintaining a low back-pressure on the column effluent, a short coil of teflon or steel capillary tubing, is often required to prohibit de-gassing of the buffers (bubble formation) in the flow-through cells. Also not indicated is the presence of a gauge, located between the mixer and injector, which serves as a more sensitive pressure detector than the continuous readout meter of the Altex pump. Since the baseline is sufficiently stable for our purposes (background "noise" equal to 0.0001 absorbance at 0.01 FSD and flow rate of 1 ml/min) no attempts to reduce pump pulsation have been made.

Column packings, buffers and solvents

Of the numerous sorbants available for peptide/small protein gradient HPLC (Table 1 A) the most useful are those possessing functional groups of low to very low polarity. The octyl (RP-8) and octadecyl (RP-18) packings of particle diameter 3, 5 or 10 μm with pore sizes ranging between 60 Å to 100 Å have found the widest applications. Samples from such widely diverse sources as tissue extracts, serum, urine, and amniotic fluid, as well as from enzymatic and chemical cleavages of isolated proteins, have been chromatographed under different conditions (Table 1 B). The buffers employed for such separations are sufficiently volatile that only lyophilization or evaporation is required to recover collected fractions. When present in high enough concentrations the salts remaining after evaporation might interfere with subsequent steps and therefore must be removed. This is most easily accomplished by simple rechromatography in a buffer which does not form salts upon drying, or by gel filtration.

As indicated in Table 1 B the limit buffer (B) contains an organic solvent which during gradient development decreases the polarity of the mobile phase. Of the solvents used their eluotropic strengths have been found to decrease in the order 1-propanol $\geq$ 2-propanol > aceto-

Table 1A: Common affinity packings used in peptide/protein HPLC

Packing type (bonded phase)	Functional group	Polarity	Solvent polarity	Elution order
nitril	$-C\equiv N$	intermediate to low	medium to high	polar → apolar
hydroxyl (diol)	$-CH-OH$ $\quad\vert$ $-CH-OH$			
phenyl	$-C_6H_5$	low		
octyl	$-(CH_2)_7-CH_3$	very low	high	
octadecyl	$-(CH_2)_{17}-CH_3$			

Table 1B: Gradient buffer systems for peptide/protein isolations on RP-8/-8 sorbants

Buffers	Source of sample chromatographed	Detection
A: 0.1 % H_3PO_4; B: CH_3CN	enzymatic digestions	UV
A: 0.1 % H_3PO_4, 0.01 M $NaClO_4$; B: 60 % CH_3CN or 2-propanol in A	tissue extracts, enzymatic digestions, chemical fragmentations	UV
A: 0.1 % TFA or HFBA; B: CH_3CN or 1-propanol in A	chemical fragmentations	UV
A: 0.5 M pyridine formate, *p*H 3.0 or 1.0 M pyridine acetate, *p*H 5.5; B: 1.0 M pyridine acetate, *p*H 5.5, 60 % 1-propanol	enzymatic digestions, chemical fragmentations, tissue extracts	fluorescence
A: 0.26 M TFA/0.21 M pyridine, *p*H 1.5; B: 60 % 1-propanol in A	as above	as above

nitrile >>> methanol. The *p*H values of the buffers, especially that of buffer A, influence the resolution of those components which weakly interact with a reverse phase support. By maintaining a low *p*H at the beginning, or throughout the gradient, the peptides chromatograph in their protonated, more apolar forms. Thus, for the more polar peptides in a mixture their resolution can be improved through adjustments in the *p*H. Since chromatography at the gradient extremes resembles isocratic elution the physical properties of the sorbant being used also influences resolution. For the isolation of quite polar peptides which are poorly separated or co-elute on 10 μm particle size packings it is sometimes possible to achieve total separation on 3 or 5 μm sorbants.

The purity of the HPLC solvents required for gradient elutions is dictated by the absorbance properties of the sample being chromatographed and the detection system available. For UV detection under 280 nm the organic solvents available as "HPLC-quality" are of absolute necessitiy. We have attempted (and presumably others) to remove the contamination in the organic solvents that binds and elutes during gradient formation but without success. The water, as well as the buffering substance employed, is of equal importance. Quartz bi-distilled water, which is first deionized, has proven to be as useful as that commercially available. The solvents used in connection with fluorescence detection, as well as for gradient development, require distillation over ninhydrin before use.

Chromatography

Prior to gradient elution it is often advisable to check sample solubility in the limiting buffer. This simple precaution will often, especially when chromatographing complex mixtures such as tissue extracts, prolong the useful operating life of a column. Additionally, it is expedient to centrifuge all samples in order to remove *any* suspended material before injection. Although we have been using the same RP-8/-18 columns without such precautions for the past 2 years, there are small, so-called guard, columns available which filter out harmful particles and strongly adsorbing compounds before they reach the analytical column.

Actual chromatography is performed at a flow rate that is a compromise between acceptable column back pressure, packing size utilized, resolution desired and the time within which a separation should be carried out. At a given flow rate, smaller diameter packings result in higher operating pressures which, through an increase in the temperature or the use of less viscous buffer, can be lowered. The stability (lability) of the substance being chromatographed often predetermines the choice of a given temperature. Thus, for buffers of low ionic strengths, containing either acetonitrile or 2-propanol, we commonly use flow rates of 0.8–1.2 ml/min; for those employed in conjunction with fluorescence detection 0.5–0.75 ml/min, both at room temperature.

Applications

Synthetic product analysis

The estimation of purity of synthetic peptides has for years depended on techniques such as TLC, microanaly-

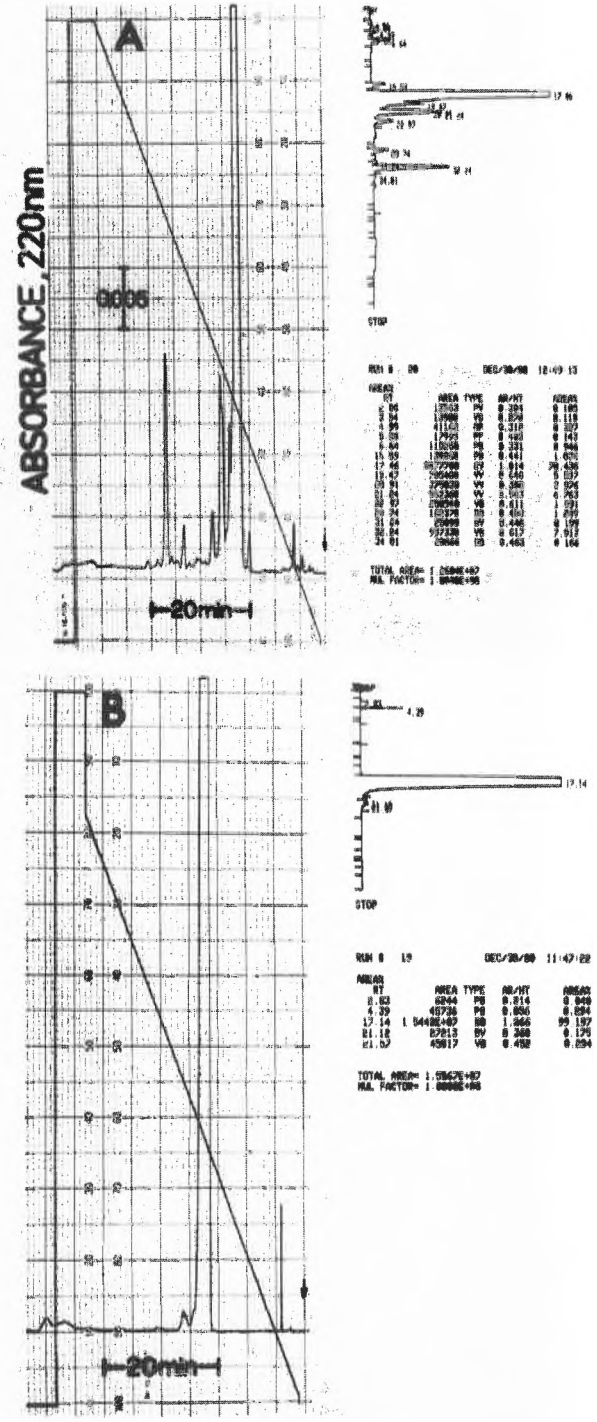

Fig. 2: Chromatography of the synthetic penta-peptide Arg-Lys-Asp-Val-Tyr. *A*, a side-product of purification (130 μg injected); *B*, final product (170 μg injected). Chromatographic system: 4.6 × 250 mm LiChrosorb RP-18 (10 μm particle size) column, flow rate 1 ml/min, room temperature. Buffers: *A*, 10 mM $NaClO_4$ in 0.1% H_3PO_4; *B*, 60% acetonitrile in *A*. Integration was performed using a Hewlett Packard 3390A reporting integrator. Recorder sensitivity was 0.05 FSD.

sis, amino acid analysis, NMR, optical characterization and, in rare instances, primary sequence determination. Through the advent of HPLC the possibility of being able to rapidly check not only peptide homogeneity, but also for possible contaminating salts or other organic matter, has been realized. As illustrated in Fig. 2 large quantities of material (> 100 μg) can be injected in order to detect small amounts of contamination. For peptides of medical interest this is of upmost importance due to the possibility of adverse side-reactions, arising not from the synthetic compound *per se* but rather, from minor contaminates from the synthesis or purification. In this example the respective areas, as well as elution times, of the eluted compounds have been determined. This in turn allows quantitative comparisons of the chromatograms and often aids in establishing if a particular step, either in the synthesis or in purification, results in an improved, more homogeneous product.

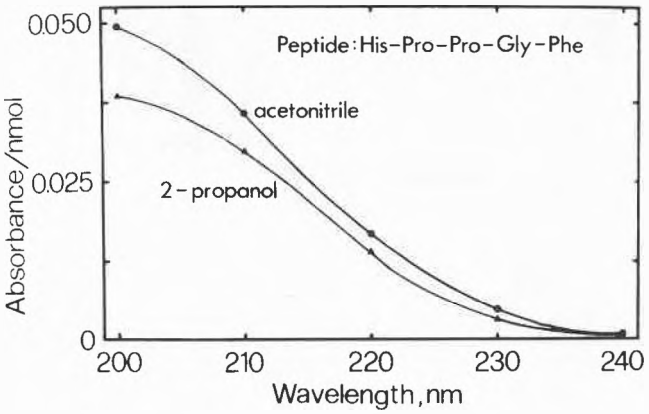

Fig. 3: The correlation between sensitivity and the absorbance properties of a peptide. A series of known concentrations were injected at the indicated wavelengths in two different buffer system: buffer *A* (in both cases identical) was as in Fig. 2; buffer *B* contained either 60 % acetonitrile or 2-propanol in *A*.

The sensitivity of reverse-phase elution of peptides is indicated in Fig. 3. Here the peptide His-Pro-Pro-Gly-Phe, a His[1]-derivative of the amino-terminus of bradykinin, has been chromatographed in two different buffer systems and the resulting peak heights (rather than areas) compared. As the results indicate sensitivity increases as the wavelength used for detection decreases. The presence of tryptophanyl and/or tyrosyl residues, as well as increased length (i. e., more peptide bonds), significantly enhances detectability. Although not indicated there is a problem associated with carrying out measurements at low wavelengths, that is the quality of chemicals, especially that of the organic solvent, used for buffer preparation. As discussed under Techniques only specially prepared chemicals, a fact reflected by their costs, are satisfactory at these lower wavelengths.

Another example of the chromatography of a synthetically derived product is shown in Fig. 4. The experimental allergic encephalitogenic (EAE) peptide, amino acid sequence Phe-Ser-Trp-Gly-Ala-Glu-Gly-Glu-Arg, can be

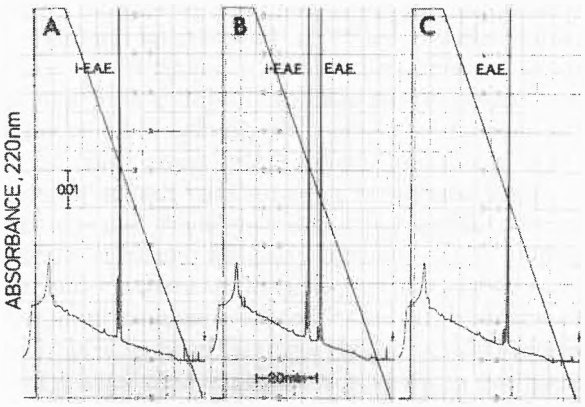

Fig. 4: HPLC of synthetic experimental allergic encephalitogenic (EAE) peptide and the p-iodo-phe[1] (i-EAE) derivative. *A*, 3.5 μg i-EAE (3.0 nmol); *B*, mixture of 3.1 μg i-EAE (2.7 nmol) and 3.0 μg EAE (2.9 nmol); *C*, 3.4 μg EAE (3.3 nmol). Chromatography conditions are as in Fig. 2.

seen to chromatograph quite differently from its iodinated derivative (i-EAE). Small amounts of contamination are also apparent in both samples which elute slightly behind each main peak. The more hydrophobic nature of the iodo group is expressed by the increased retention times of the i-EAE derivative. This particular example indicates (i) the high resolution capabilities of reverse-phase gradient HPLC and (ii) that the major contributing factor in separation is the hydrophobicity of the individual components being chromatographed.

Natural product analysis

For the same reasons as discussed in the preceeding section it is necessary to control the homogeneity of products isolated from natural sources. In Fig. 5 the chromatography of two commercially available porcine insulins is illustrated. The amounts, injected directly

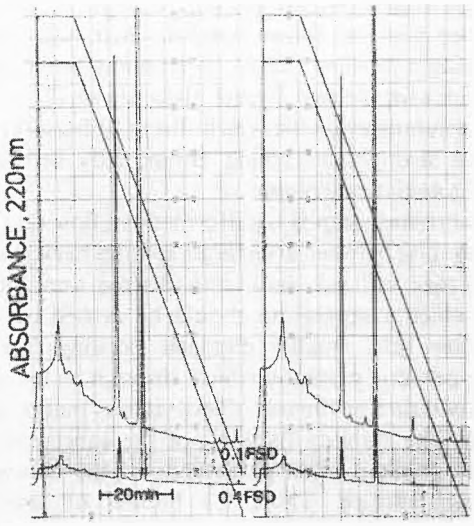

Fig. 5: Gradient elutions of insulin preparations. Injected was a volume containing 3.3 μg; elution was carried out as in Fig. 2. For each sample duplicate injections were performed at FSD 0.1 (upper) and 0.4 (lower).

from the original containers and concentrations calculated from the labels on each product, were 3.3 μg ($\sim$ 600 pmol). Since the smaller of the two peaks from each preparation elutes at a constant 76% of buffer B, and since their areas were roughly equal, it was tentatively identified as insulin. This was subsequently confirmed by collection of the peaks and amino acid analysis (yields > 70%). The remaining large peaks probably represent the preservatives m-cresol and methyl-p-oxybenzoate.

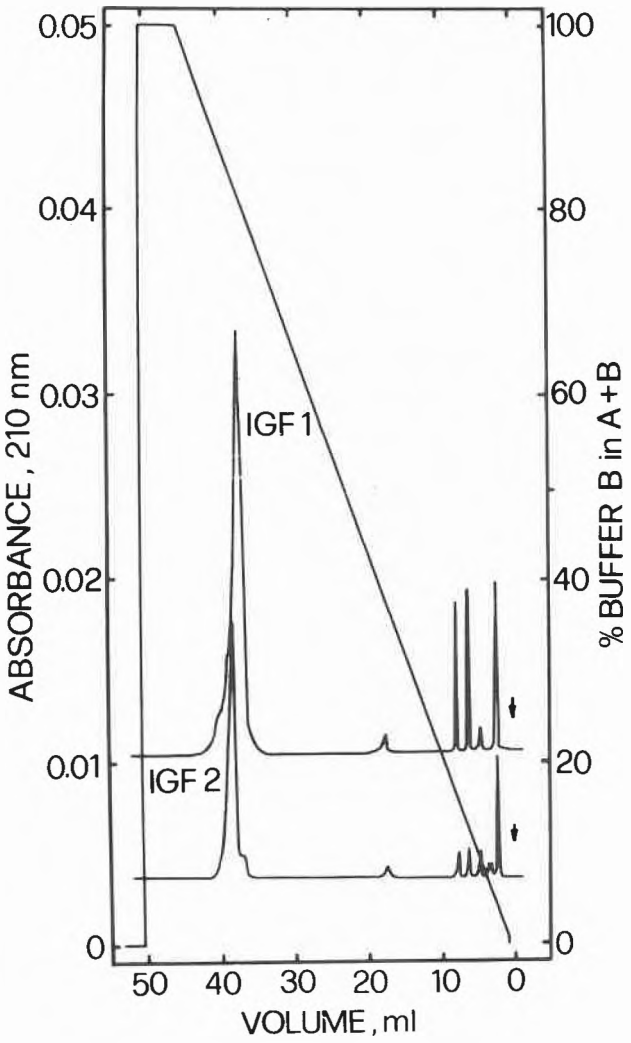

Fig. 6: HPLC of human insulin-like growth factors (IGF) 1 and 2. Injected was $\sim$ 5 μg of each polypeptide; recorder sensitivity of 0.05 FSD. Elution was carried out as in Fig. 2. The elution points for IGF-1 and -2 were 81% and 83% of buffer *B*, respectively.

The HPLC analyses of the polypeptides IGF-1 and -2 are illustrated in Fig. 6. The results have been redrawn, rather than photographed directly, since at the wavelength used for detection (210 nm) the significant background due to the elution buffer itself complicates clear-cut interpretation. The small retention differences between the factors allow separation of the two components. They have also been isolated from other similar (with respect to size and electrophoretic properties) polypeptide mixtures. The series of smaller peaks observed eluting at the beginning of the gradient derive from purification and represent non-peptide material.

Post translational modifications

A clinical application of HPLC is demonstrated by the measurement of the glycosylated N-terminal component (HbA_{Ic}) from human haemolysates. Fig. 7A, shows the

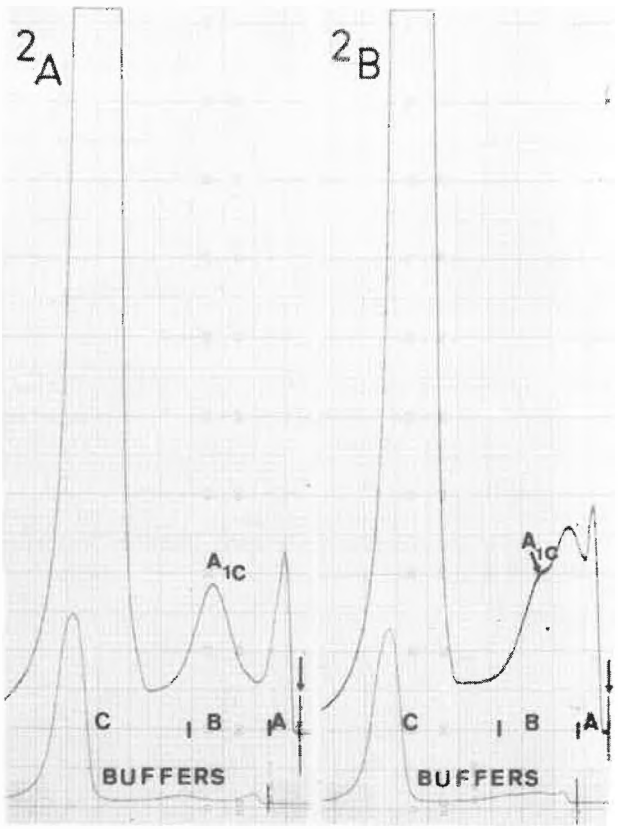

Fig. 7: Analysis of human haemolysates by HPLC on Bio-Rex 70. *A*, Haemolysate incubated at 37°, under CO for 2 days. *B*. Haemolysate incubated as in *A*, with the addition of 50 mM glucose. Chromatography was on Bio-Rex 70 (minus 400 mesh) packed in a stainless steel column (4.6 × 250 mm), eluted at room temperature with phosphate buffers of increasing ionic strength and decreasing *p*H. Flow rate was 1 ml/min and absorbance was measured at 410 nm; 20 μl of haemolysate injected.

complete separation of HbA_{Ic} from the fast running haemoglobins and the major component of HbA_O. The use of HPLC techniques permits rapid analysis of numerous haemolysates and as a method for the screening of diabetes should be considered. Demonstrated in Fig. 7B is the high-performance, in comparison to classical liquid chromatography, of this technique. Here the analysis is of a haemolysate incubated with additional glucose, the partial separation of a 'fast' HbA_{Ic} is clearly evident. To date the occurrence of an intermediate in the formation of HbA_{Ic} has been demonstrated only by isoelectric focussing.

Toxological testing of herbicides and pharmaceuticals is of utmost importance for our society. An example of the

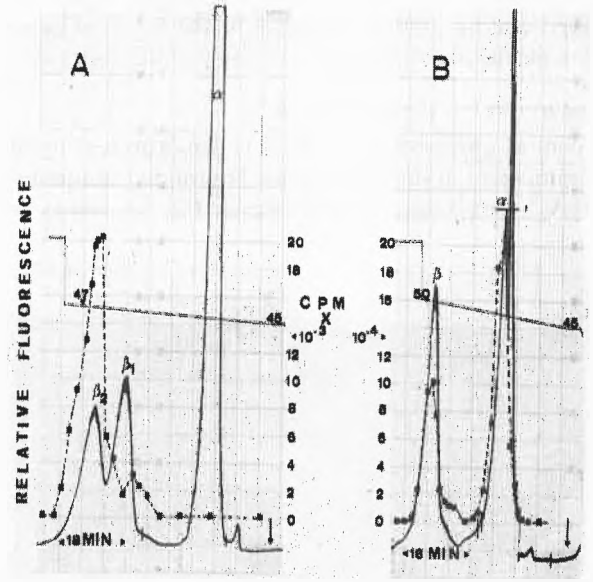

Fig. 8: Analysis of simetryn sulfoxide modified rat haemoglobins by reverse phase HPLC. *A*, ^{14}C simetryn sulfoxide followed by cold iodoacetamide, modified globins; *B*, ^{14}C iodoacetamide modified globins. A column (4.6 × 250 mm) containing LiChrosorb RP-8 was eluted at a flow rate of 0.65 ml/min with pyridine formate buffers *p*H 3.0. The gradient of increasing 1-propanol is shown for % buffer B (containing 60 % v/v 1-propanol). Detection was by on-line fluorescence detection (OPA) of 2 % of the column effluent.

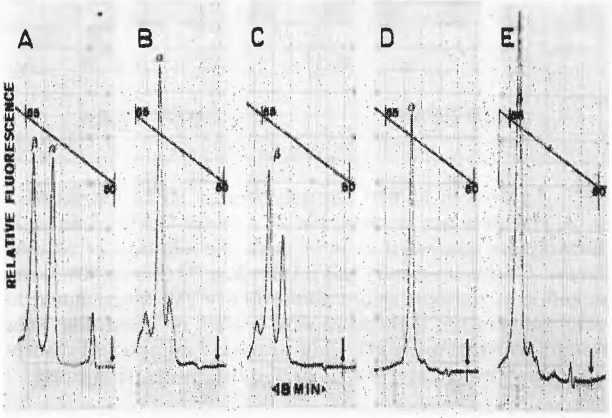

Fig. 9: Preparative isolation and analysis of rat globin chains by reverse phase HPLC. *A*, Preparative isolation (50 nmol); *B* and *C*, Analysis on 10 % of α and β chains separated in *A*; *D* and *E*, α and β chains separated in *A*, after reduction. Elution was with trifluoroacetic acid, pyridine buffers (*p*H ca. 1.5), other conditions were as in Fig. 8.

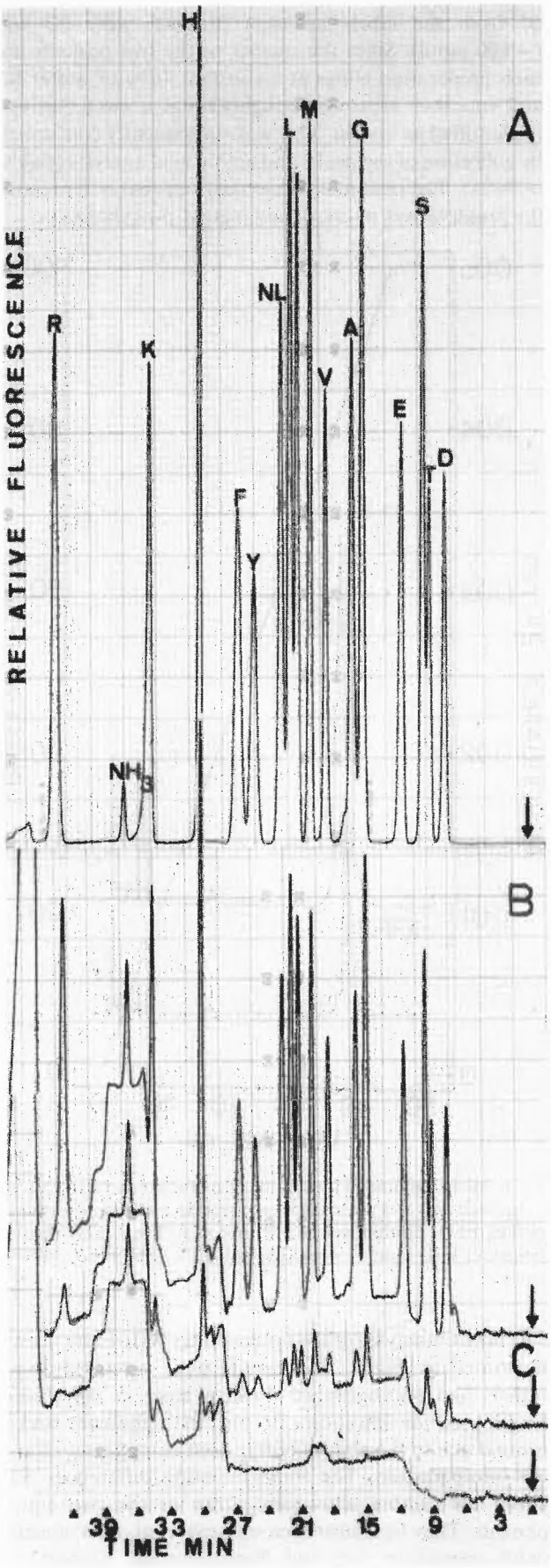

Fig. 10: Standard analyses of amino acids normally found in protein hydrolysates. *A*, 10 nmol per component: *B*, 100 pmol: *C* 10 pmol. DC 4A (Durrum) resin in a stainless steel column (3.2 × 250 mm) was eluted sequentially with buffers containing 0.2 N Na^+ (*p*H 3.25), 0.2 N Na^+ (*p*H 4.25) and 1.1 N Na^+ (*p*H 7.9). Detection was by fluorescence, using post-column derivatization with OPA.

power of HPLC for evaluating, at the molecular level, possible toxological effects, was given by our study on the reaction of metabolites of the herbicide, simetryn, with rat haemoglobins. In Fig. 8 the separation by reverse phase HPLC of rat V globin chains is shown, Haemoglobins modified with radioactively labelled simetryn sulfoxide gave three peaks (Fig. 8A) compared to unreacted ones which gave only two (Fig. 8B). Subsequent characterization (using virtually only HPLC) confirmed that rat haemoglobins bound one sulfoxide on one of the two β chains within the haemoglobin tetramer. A complete separation of β_2 (the chain carrying the label) from β_1 was achieved even though substitution was at only one position in a molecule containing ca. 150 amino acid residues. The reactive amino acid residue within the β chain is present only in the haemoglobins from rat.

Detection of chemical heterogeneity

It is well recognized that a symmetrical elution peak, as in any other chromatographic system, does not necessarily represent a pure compound. The converse, i.e. multiple peaks from a compound that to all intents and purpose is pure, can arise when polypeptides are analyzed by HPLC. An example for rat globin chains is shown in Fig. 9. The chains, α and β, were preparatively separated by reverse phase HPLC (Fig. 9A), dried under vacuum and a portion of each re-injected (Figs. 9B and C). Multiple peaks were obtained for each chain, however after reaction with a sulfide reducing agent the purity of each fraction became apparent (Figs. 9D and E). Indication of impurity arose only because of disulfide bond formation.

Amino acid analysis

An important analysis, in both research and clinical laboratories, is that of amino acids. As a result of our use, exclusively of HPLC for analytical or preparative separation of peptides/proteins and the analysis of amino acid derivatives, we constructed an amino acid analyzer using only HPLC components. The performance, illustrated in Fig. 10, demonstrates the usual attributes of HPLC, i.e. rapid analysis time, high reproducibility, and analysis capabilities at either high or low sensitivities.

Acknowledgements

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