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Separation and tryptic digest mapping of normal and variant haemoglobins by capillary electrophoresis”

Gordon A. Ross

Medical Professorial Unit, Medical College of St. Bartholomew's Hospital, West Smithfield, London ECIA 7BE (UK)

Peter Lorkin

Department of Haematology, St. Bartholomew's Hospital. West Smithfield, London EC1A 7BE (UK)

David Perrett*

Medical Professorial Unit, Medical College of St. Bartholomew's Hospital, West Smithfield, London EC1A 7BE (UK)

ABSTRACT

Characterization of haemoglobin (Hb) through whole protein separations and tryptic digest mapping allows the identification of structural Hb variants which result in haemoglobinopathies. Tryptic digest mapping by conventional two-dimensional paper chromatograph–electrophoresis provides high resolution but requires 48 h, while gradient elution reversed-phase high-performance liquid chromatography (HPLC) is faster (1.5 h), there is decreased resolution. CE analysis provides a fast separation with high resolution. We have used CE to optimise the tryptic digestion of globin purified from normal human haemoglobin A and to analyze tryptic digests from normal Hb. The separations were optimised and peak identification performed using UV scanning detection. In the optimised tryptic digest separation up to 28 peaks could be resolved in < 20 min. These peaks were identified as far as possible and a high-resolution map of the digest was constructed. The optimised analytical conditions were used to observe the separation pattern obtained from normal adult haemoglobin (HbA), common variant haemoglobins and some rarer haemoglobin variants.

INTRODUCTION

Characterisation of haemoglobin (Hb) through whole protein separations and sequence analysis allows the identification of clinically important Hb variants. These haemoglobinopathies may be divided into (a) synthesis disorders (the thalassaemias) and (b) structural variations. Most of the 400+ structural variants known to date are single amino acid substitutions [1]. Confirmation and characterisation of a variant may be done by tryptic digest mapping and amino acid analysis. Tryptic digestion usually gives 29 peptides from normal HbA; four are insoluble and lost during the digest preparation and two are single amino acids. Substitutions which alter the number and/or position of cleavage points affect the number and size of the peptide products; substitution of amino acid residues within the peptides may be detected if they change those characteristics of the peptide upon which the selectivity of the separation technique is based [i.e. charge for electrophoresis or hydrophobicity for high-performance liquid chromatography (HPLC)].

Since the 1950s, two-dimensional paper chromatography-electrophoresis has been used to separate Hb tryptic digests [2] and although taking about 48 h, gives a very high resolution of the peptides. Gradient elution reversed-phase HPLC has been avail-

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*Corresponding author.

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able for tryptic digest mapping since the late 1970s [3], giving a marked improvement in the analysis time to approximately 1 h, however, since HPLC lacks the resolving power to uniquely separate all peptides it has not yet gained a major foothold in clinical Hb fingerprinting.

Capillary electrophoretic (CE) analysis of proteins and peptides has been investigated [4,5] and has been applied to the characterisation of biosynthetic human growth hormone [6–8], the analysis and micropreparation of peptides from calcitonin using ^2H_2O-based buffer solutions [9] and in the mapping and map prediction of tryptic digests of myoglobin [10]. The low sample requirements for CE have been exploited by developing micro techniques for tryptic digestion needing only ng amounts of protein [11,121. CE provides an orthogonal separation to reversed-phase HPLC and, in combination, can be used to provide a means for detecting single residue substitutions [13]. Detection is generally by UV absorbance although derivitisation of arginine containing peptides to give a fluorescent moiety [12] reduces the number of peptides observed and gives a less crowded map.

CE analysis of tryptic digests of haemoglobin P-chain have been investigated by Ferranti *et al.* [14] who compared this with the β-chain digests of variants in order to identify the affected peptides; these were confirmed using fast atom bombardment mass spectrometry (FAB -MS) analysis of the tryptic digest although the CE separation was not interfaced with MS. Haemoglobinopathies have also been investigated using CE separation of whole globin chains [15,161 and this provides a simple method of identifying which sub-unit chain is involved in the haemoglobinopathy.

Among our initial studies on the applications of CE in the bioanalytical laboratory, we have investigated its application to the analysis of normal and variant haemoglobins and have attempted to provide a peptide map from the CE analysis of tryptic digests of HbA.

MATERIALS AND METHODS

Methanol, acetone, 2-propanol and acetonitrile was HPLC grade (Rathburn, Walkerbum, UK). Trifluoroacetic acid (TFA), sodium dodecyl sulphate (SDS), NaH_2PO_4, heptanesulphonic acid (HSA), octanesulphonic acid (OSA) NaOH and HCl were all AnalaR grade and were obtained from BDH (Poole, UK). Trypsin [L-1-tosylamide-2-phenylethyl chloromethyl ketone (TPCK) treated] was supplied by Sigma (Poole, UK). Water was distilled and deionised prior to use in buffers. Fused-silica capillaries were obtained from Polymicro Technologies (Composite Metal Services, Hallow, UK).

Preparation of Hb

Whole blood (10 ml) was collected into a tube containing lithium heparin and spun for 15 min at 2000 g. The supernatant was aspirated and the buffy coat removed. The remaining red cells were washed with 10 ml of isotonic saline (NaCl, 0.9%, w/v) and the suspension spun and aspirated as before (3 ×). The cells were diluted 1:10 with distilled water to lyse them, and spun at 10 000 g for 10 min to sediment cell debris and any other solid material. The remaining solution had a concentration of approximately 2 mg Hb/ml. The supernatant was then concentrated to approximately 5 mg Hb/ml by ultrafiltration using a Minicon B15 unit (Amicon, Stonehouse, UK).

This concentrate was stirred into cold acid acetone (conc. HCl–acetone, 2:98, v/v) to precipitate globin and spun at 2000 g for 10 min. The supernatant was removed and the globin washed with excess acetone then spun and aspirated as before. The final precipitate freeze dried before tryptic digestion. Isolation of α and β chains prior to their tryptic digestion was performed using ion-exchange gel chromatography on CM-Sepharose CL-6B [3].

Time course of tryptic digestion

Tryptic digestion was performed by incubating 1 volume of globin (10 mg/ml water) with 0.2 volume of 0.5 *M* NH_4HCO_3, 0.1 volume of 2 mg/ml water trypsin (TPCK treated) and 0.1 volumes of 7 m*M* tryptophan (internal standard) at 37°C. Samples were taken every 10 min for 1 h, then every 20 min for a further 80 min. The digestion was stopped by collecting the aliquot into an equal volume of 0.5 *M* CH_3COOH and placing it in hot water for 2-3 min to precipitate insoluble peptides. Tryptic digest of α and β chains and of other Hbs were performed using the above procedure. The tryptic digest was freeze dried then reconstituted in water. The con-

centration of the tryptic digest sample was approximately 7 mg/ml. Routinely digestion was performed for 2 h.

CE equipment

Separations and detection were performed using a SpectraPHORESIS 1000 (Spectra-Physics, Hemel Hempstead, UK). This instrument allows single and multi-wavelength detection and also high-speed scanning in the UV or visible ranges. Capillaries of 50 μm were wound onto capillary cassettes for use in the instrument; the lengths used were either 44 cm (37 cm to window) or 70 cm (63 cm to window). All samples were loaded hydrodynamically. The load volume was approximately 5-10 nl.

RESULTS AND DISCUSSION

Buffer pH, running voltage and temperature were first optimised to give a separation within a time of approximately 20 min. Further modifications including the addition of organic modifiers and surfactants were investigated in an attempt to improve upon the initial separations. Individual peptides were obtained from either preparative HPLC [3] or from conventional two-dimensional separation by paper chromatography and electrophoresis.

Optimisation of tryptic digest separation

Buffers of pH 2.5, 6.0 and 10.0 were constructed and used with a running voltage of 25 kV. In manipulation of an electrophoretic separation buffer pH is one of the most important parameters available. Electrophoresis at all pH values allowed a completed separation in less than 20 min, however, pH 6 and 10, when the electroosmotic flow is significantly greater [17], gave much poorer separation than that obtained at pH 2.5. At this low pH most of the peptides should be positively charged. Further manipulation of the pH from 1.5 to 3.00 gave no further enhancement of the separation obtained.

At pH 2.5 the buffer concentration was 50 m*M* giving a current of < 50 μA. Running voltages of 20, 25 and 30 kV were explored with temperatures of 20 to 40°C. A resolution map [18] was constructed for those pairs of peaks which exhibited poor resolution. Maximum resolution of the digest was observed at 25°C using a voltage of 25 kV.

Further studies were performed using the following operating conditions: buffer: 50 m*M* H_3PO_4 adjusted to pH 2.5 with 1 *M* NaOH; run: 25 kV, 25°C; capillary: 70 cm (63 cm to detection window) × 50 μm I.D.; detection: UV absorbance at 200 nm or scanned from 200 to 300 nm.

The separation obtained under these conditions provided good resolution for many of the peaks. However in the central area of the electropherogram (time 12-14 min) there are a number of peaks migrating very closely together. Buffer additives were used in an attempt to increase the resolution in this area.

The effect of buffer additives

Organic modifiers were added to the running buffer over the range 1 to 20% (v/v). 2-Propanol, a useful modifier in HPLC of peptides, caused a marked increase in analysis time which was accompanied by a loss of resolution. Acetonitrile addition resulted in poorer peak shape among the faster moving peptides and addition of methanol caused decrease in resolution and a slightly faster run time. In all no benefits were obtained from adding organic modifiers.

TFA, which can ion-pair with peptides, was added at concentrations of 1 and 10% (v/v). At the higher concentration the amount of added 6 *M* NaOH required to re-establish the buffer pH to 2.5 resulted in very high currents and frequent short circuits while at 1% (v/v) TFA a marked increase in baseline noise obscured many peaks. SDS was added to the buffer at concentrations of 1, 5 and 50 m*M*. Both 1 m*M* and 5 m*M* concentrations are below the critical micellar concentrations (CMC) for SDS in water and in salt concentrations of < 0.05 *M*, therefore at these concentrations SDS will act as an ion-pairing agent. At the higher concentration of 50 m*M* micellar formation will occur. At concentrations of 5 and 50 m*M* no peaks were observed at all and there was a marked reduction in the number of peaks observed at 1 m*M*. Other ion-pairing agents such as octane-sulphonic acid and heptane-sulphonic acid contributed nothing to the resolution of the mixture and often served only to increase the current.

HbA separation

There are, theoretically, 29 peptide products of HbA tryptic digestion, four of these, αT12, αT13,

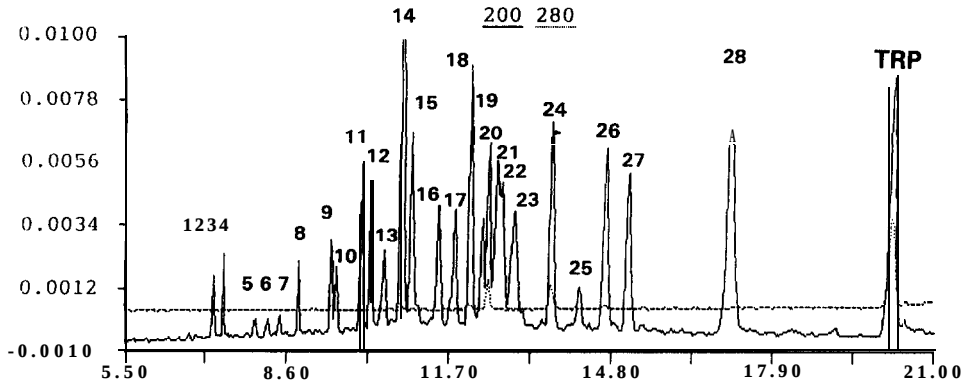

Fig. 1. CE separation of tryptic digest of purified globin from normal haemoglobin A. Conditions as in text. Solid line, absorption at 200 nm; broken line, absorption at 280 nm. x-Axis is migration time (min); y-axis is absorbance units.

βT10 and βT12 are insoluble and precipitate out during the digestion preparation. Two other single lysine amino acid products, αT8 and βT8, are not seen with the UV detection system which mainly measures peptide bond absorption at ca. 200 nm. There are further peptides generated from incomplete digestion e.g. αT8-9, or in the case of βT5-ox from the oxidation of a methionine group. Optimum separation conditions should therefore give an electropherogram containing a minimum of 23 peaks. The optimised separation gave 26 peaks with a mean efficiency (N) of 83 000 plates (range 50 000-130 000) (Fig. 1). The relative standard deviation for retention times varied from 0.38 to 1.04% with a mean of 0.67% ($n = 6$).

Optimisation of tryptic digestion

The enzymic reaction was monitored at the stated

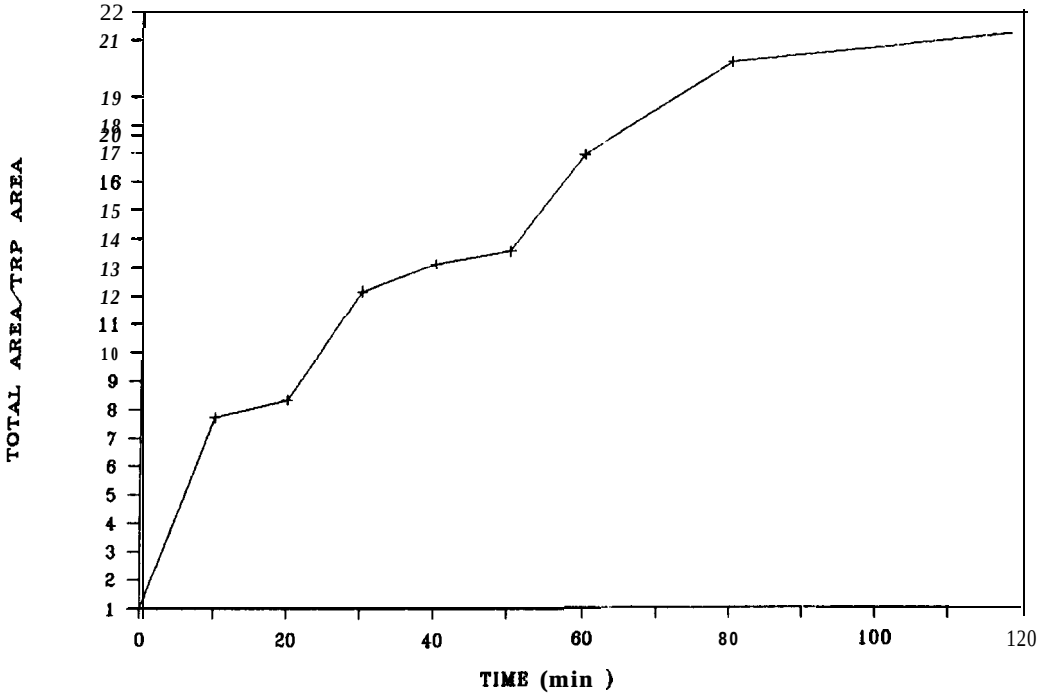

Fig. 2. Total spatial area relative to tryptophan (internal standard) over tryptic digestion time course.

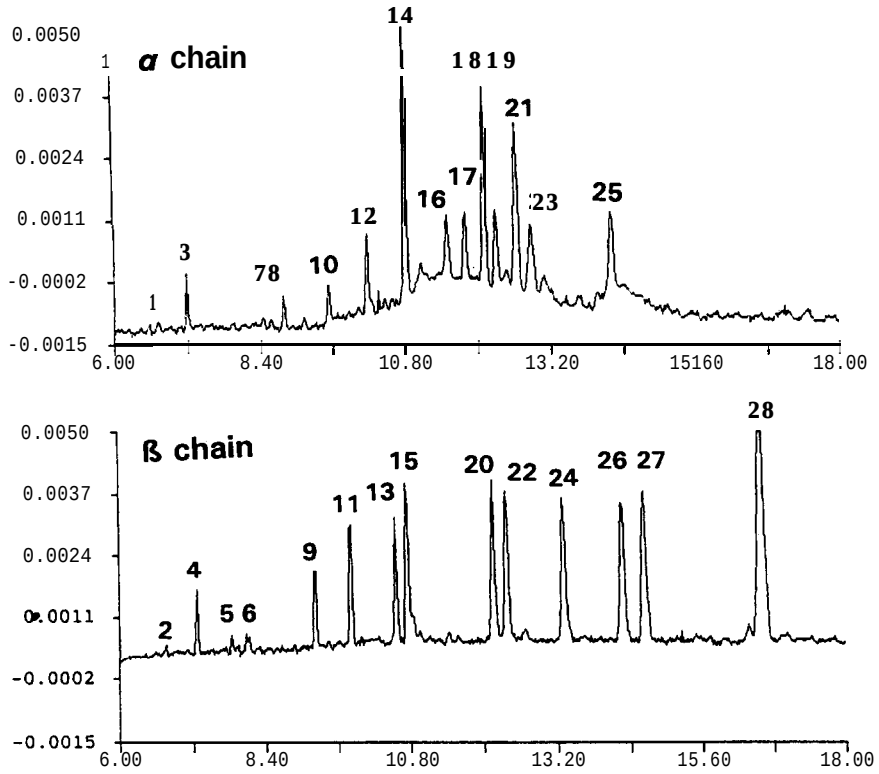

Fig. 3. CE separation of tryptic digest of individual a and β chains and peak assignment to subunit source. Conditions as in text. x-Axis is migration times (min); *y*-axis is-absorbance units at 200 nm.

time intervals for the number of peaks and total peak area relative to tryptophan (internal standard). The reaction time was optimised with regard to the number and relative area of peptides produced. The number of peaks observed increased from 0 to 29 within 10 min, however a plot of total peak area/tryptophan area indicated that optimum digest generation was achieved at time 2 h (Fig. 2). A control digest containing no globin showed no peaks until time 140 when some minor peaks appeared probably due to autodigestion of trypsin.

STRATEGIES FOR PEAK IDENTIFICATION

Further elucidation of any tryptic digest map involves identifying the peaks as specific peptide products of the digest. This can be performed by amino acid analysis of isolated peptides but since HbA is well characterised the peptide products are known. Comparison of electropherograms from tryptic digests of whole HbA with those from digests of purified *a* and *β* chains can identify the subunit source of the peaks in Fig. 3. Since the peptide sequences are known it is possible to predict a number of characteristics of the peptides which would correspond to particular peaks, e.g. absorbance at 280 nm for peptides with aromatic residues (Tables I and II).

Spectral characteristics

Rapid scanning detection can be used to help assign known peptide structure to separated peaks. Corrected peak areas ***(i.e.*** area/migration time or spatial area) at 200 nm should be proportional to the number of peptide bonds in a peptide, although this may be larger where there are aromatic side groups on amino acid residues contributing to the absorbance at 200 nm. These same peptides containing tryptophan, tyrosine or phenylalanine also exhibit typical absorbance characteristics at around

TABLE I

α CHAIN PEPTIDES FROM TRYPTIC DIGESTION OF PURIFIED GLOBIN

Peptides containing Trp and Tyr (bold face) can be expected to absorb in the range 280–300 nm

Peptide	Amino acid residues	Sequence	Absorbance at 280 nm	Peak No.
UT1	7	Val-Leu-Ser-Pro-Ala-AspLys	None	
αT2	4	Thr-Asn-Val-Lys	None	
αT3	5	Ala-Ala-TrPGly-Lys	Strong	12
αT4	15	Val-Gly-Ala-His-Ala-Gly-GlyGlu-TyrGly-Ala-Glu-Ala-Leu-Leu-Arg	Weak	14
αT5	9	**Met–Phe–Leu–Ser–Phe–Pro–Thr–Thr–Lys**	None	
aT6	16	**Thr–Tyr–Phe–Pro–His–Phe–Asp–Leu–Ser–His–**Gly-Ser-AlaaGln-Val-Lys	Weak	18
aT7	4	**Gly–His–Gly–Lys**	None	
αT9	29	**Val–Ala–Asp–Ala–Leu–Thr–Asn–Ala–Val–Ala–His–Val–Asp–Asp–Met–Pro–Asn–Ala–Leu–Ser–**Ala-Leu-Ser-AspLeu-His-Ala-His-Lys	None	
αT10	2	Leu-Arg	None	7
αT11	7	Val-AspPro-Val-Asn-Phe-Lys	None	
aT14	2	Tyr-Arg	Weak	8

240-300 nm. Phenylalanine residues absorb weakly at these wavelengths and have a cut-off at around 270 nm, therefore peaks absorbing at 280–300 nm contain either tyrosine or tryptophan residues. These can be further subdivided since the absorbance by tryptophan containing peptides will be very much larger than tyrosine containing peptides because of their differing molar absorptivities (5000 $M^{-1}\,cm^{-1}$ and 1000 M^{-1} cm-' respectively). Further differentiation may be gained by observing the UV cut off since tyrosine residues have a cut off at around 290 nm whereas tryptophan residues cut off at > 310 nm.

TABLE II

β CHAIN PEPTIDES FROM TRYPTIC DIGESTION OF PURIFIED GLOBIN

Peptide	Amino acid residues	Sequence	Absorbance at 280 nm	Peak No.
βT1	8	Val-His-Leu-Thr-Pro-GluGlu-Lys	None	11
βT2	9	Ser- Ala-Val-Thr-Ala-Leu-Trp-Gly-Lys	Strong	20
βT3	13	**Val–Asn–Val–Asp–Glu–Val–Gly–Gly–Glu–Ala–Leu–Gly–Arg**	None	27
βT4	10	Leu-Leu-Val-Val-Tyr-Pro-TrkThr-Gln-Arg	Strong	24
βT5	19	**Phe–Phe–Glu–Ser–Phe–Gly–Asp–Leu–Ser–Thr–Pro–Asp–Ala–Val–Met–Gly–Asn–Pro–Lys**	None	28
βT6	2	Val-Lys	None	
βT7	4	Ala-His-Gly-Lys	None	
βT9	16	**Val–Leu–Gly–Ala–Phe–Ser–Asp–Gly–Leu–Ala–**His-Leu-Asp-Asn-Leu-Lys	None	
βT11	9	Leu-His-Val-AspPro-Glu-Asn-Phe-Arg	None	13
βT13	12	**Glu–Phe–Thr–Pro–Pro–Val–Gln–Ala–Ala–Tyr–**Gln-Lys	Weak	
βT14	12	Val-Val-Ala-Gly-Val-Ala-Asn-Ala-Leu-Ala-His-Lys	None	15
βT15	2	**Tyr–His**	Weak	9

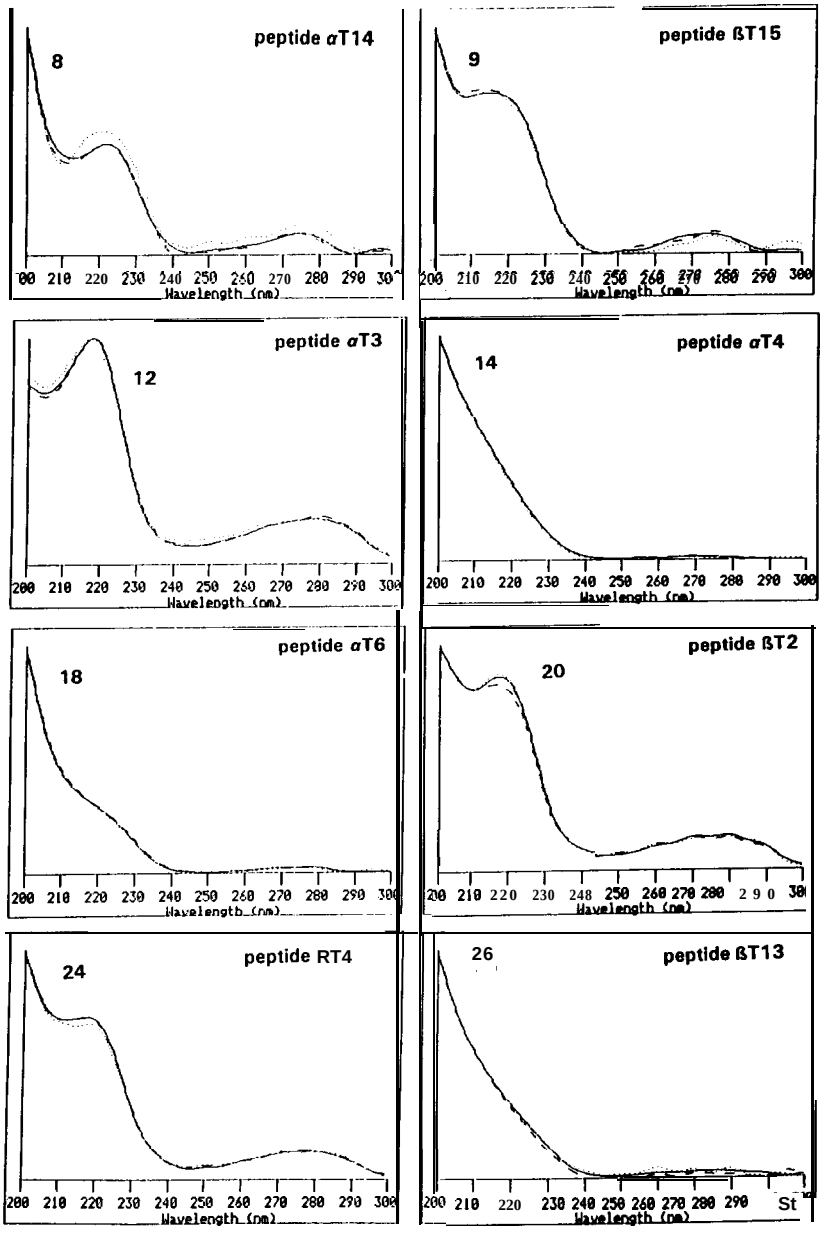

Fig. 4. Spectra of aromatic side chain containing peptides. Peak number top left of each spectra and corresponding peptide in top right.

From the known peptides of HbA it can be predicted that 8 peptides should absorb at 280 nm (Tables I and II), further, the tryptophan-containing peptides should absorb more strongly than those containing tyrosine. Therefore αT3, βT2 and βT4 should absorb strongly and αT4, αT6, αT14, βT13 and βT15 should absorb weakly at 280 nm. This is borne out by observing 8 peaks, 3 large and 5 smaller ones, at 280 nm. From Fig. 3 which identifies the subunit source of the peaks and by observing the peak spectra, those peaks which contain tryptophan and those which contain tyrosine can be assigned

TABLE III

PEAK ASSIGNMENT TO α OR β GLOBIN CHAINS

Sub unit	Peaks (numbered from Fig. 1)
α	
β	2, 1, 4, 3, 7, 5, 8, 6, 9, 10, 11, 12, 13, 14, 15, 16, 20, 17, 22, 18, 24, 19, 26, 21, 27, 23, 28 25

since tyrosine containing peptides have a cut off lower than 300 nm whilst those containing tryptophan have a cut-off higher than 300 nm (Fig. 4). Further, correlation between the spatial peak areas at 200 nm and the known number of peptide bonds allows the assignation of peptides αT3, αT4, αT6 and αT14 to peaks 12, 14, 18 and 8 respectively, and peptides βT2, βT4, βT13 and βT15 to peaks 20, 24, 26 and 9 respectively.

Standard addition of isolated peptides

Isolated peptides from two-dimensional paper chromatography and electrophoresis or HPLC were freeze-dried and reconstituted in 100 μl water, then combined with 1 m*M* tryptophan (1: 1, v/v) and run to determine their individual migration times and relative to tryptophan. Tryptic digests of isolated α and β chains were treated similarly and the relative migration times of the peaks recorded. The sub-unit tryptic digests were then spiked with isolated peptides and observed for increased absorption at 200 nm of any one peak. Comparing relative retention times and increased peak areas in spiked samples allowed us to assign 13 peaks to specific peptides (Tables III and IV). Impurities in the fractions from either HPLC or two-dimensional paper chromatography and electrophoresis believed

TABLE IV

COMPARISON OF CE WITH BOTH HPLC AND TWO-DIMENSIONAL PAPER CHROMATOGRAPHY AND ELECTROPHORESIS FOR Hb TRYPTIC DIGESTS

Method	Sample volume	Time	Resolution	Automation
Two-dimensional paper chromatography and electrophoresis	200 μl	48 h	Very high	No
Gradient elution reversed-phase HPLC	20 μl	75 min	Good	Yes
Capillary electrophoresis	10 nl	< 25 min	High	Yes

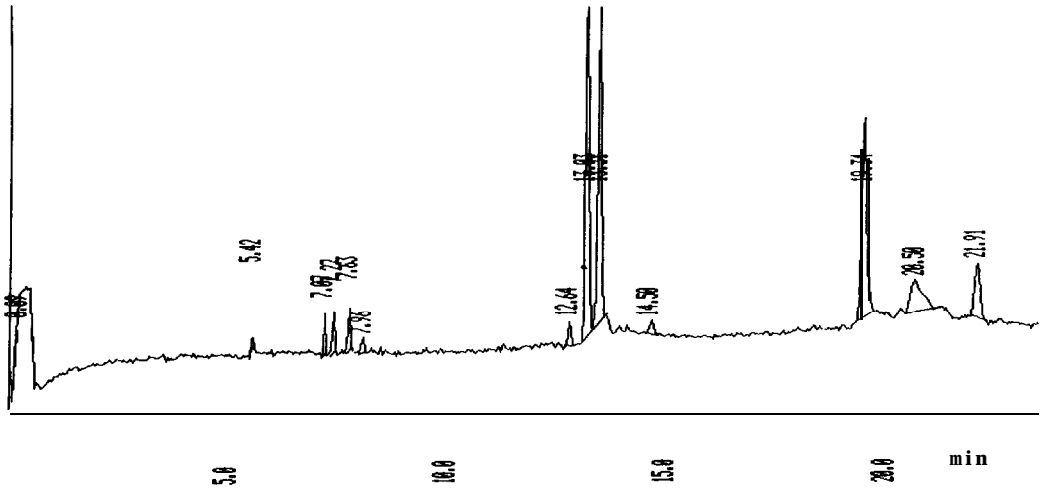

Fig. 5. CE separation of preparative HPLC fraction corresponding to HbA peptide aT5. Conditions as in text. Wavelength: 200 nm; 0.009 AUFS.

to contain isolated peptides hindered the complete mapping of the Hb tryptic digest. The electropherograms obtained from such fractions show a number of peaks with no indication of what is impurity and what is peptide (Fig. 5).

HAEMOGLOBIN VARIANTS

In order to identify the presence of a structural variant, the tryptic digest separation pattern must be reproducibly specific for that variant and different from the normal pattern.

HbE

HbE is probably the most common Hb variant in the world. It results from the substitution of glutamic acid by lysine at position 26 in the B-chain of affected individuals. The consequences of this are

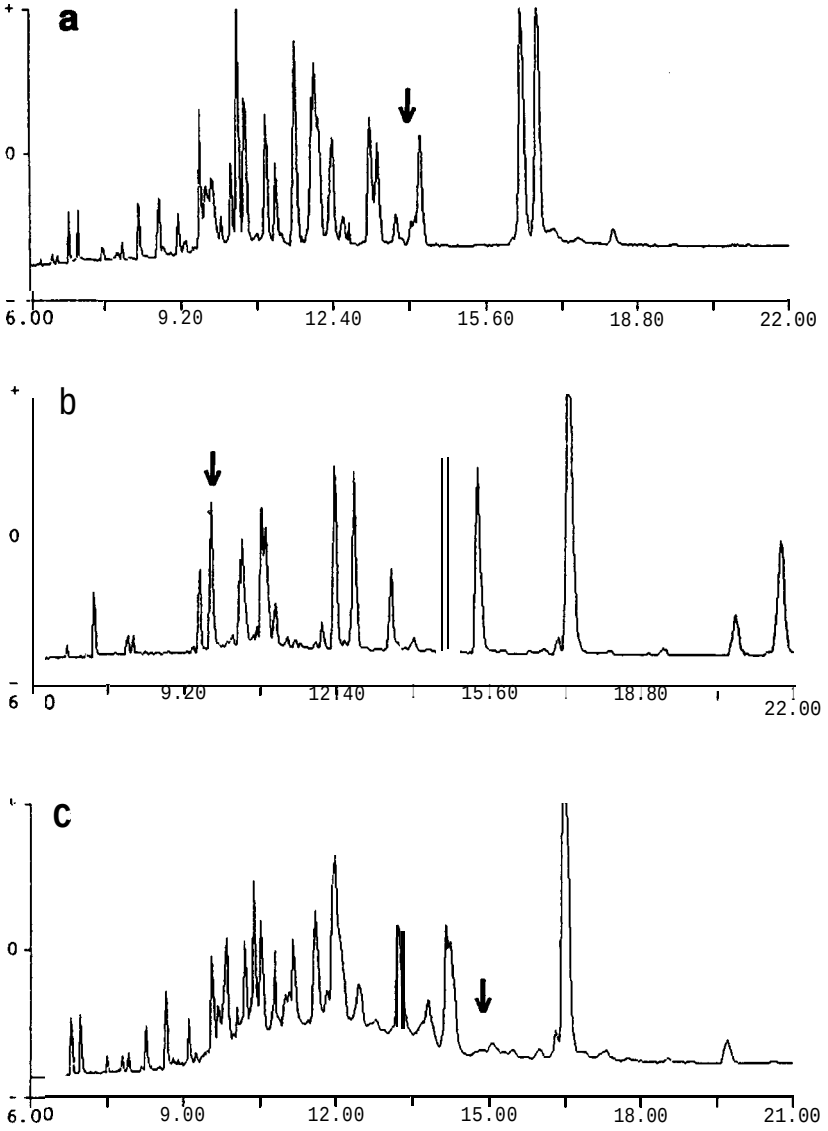

Fig. 6. (a) CE separation of digest of HbE. Conditions as in text. Arrow indicates position of missing peptide. (b) CE separation of digest of purified HbβS. conditions as in text. Arrow indicates affected peptide βT1. (c) CE separation of digest of HbO Arab. Conditions as in text. Arrow indicates position of missing peptide. x-Axis is migration times (min); y-axis is absorbance units normalised to the largest peak.

that the peak corresponding to βT3 should be missing in the homozygote and reduced in the heterozygote to be replace by two peptides corresponding to βT_{18-26} and βT_{27-30}. Fig. 6a shows the separation of HbE tryptic digest indicating the absence of peak 27 which corresponds to the affected peptide.

HbS

HbS is the cause of sickle cell disease in the homozygote (HbSS) and sickle cell trait in the heterozygote (HbAS). The amino acid substitution occurs at position 6 in the β chain of affected individuals. In this case the glutamic acid is replaced by a valine residue. The overall charge on the peptide (βT1) should therefore be less negative. This is reflected in Fig. 6b which indicates the position of the affected peptide. In this case the peptide has a different mobility compared with normal and subsequently its position in the electropherogram has changed. In this instance the altered mobility of this peak identifies it as peak 11 in the separation of HbA tryptic digest.

HbO-Arab

HbO-Arab is a rare haemoglobinopathy which results from the substitution of glutamic acid at position β_{121} by lysine. This results in the truncation of βT13 from 11 amino acids to 10 amino acids since the glutamic acid at β_{121} is the first amino acid in normal βT13. This peptide has already been identified as peak 26 and it can be seen in Fig. 6c that the peak is indeed missing from the digest.

CONCLUSIONS

In terms of sample volume CE uses much less than either HPLC or two-dimensional paper chromatography and electrophoresis (Table IV), however in this application this is quite irrelevant since even with analysis of infant haemoglobin there is no sample limitation. However in the case of bioactive peptides manufacture in the pharmaceutical industry this aspect becomes very important. CE outstrips any of the methods in terms of speed of analysis but despite providing very high resolution it is not at present as good as the two-dimensional method. Combination of the CE separation with HPLC analysis of tryptic digest could however provide a resolution markedly improved over that obtained from conventional two-dimensional paper chromatography and electrophoresis.

Quantitative aspects of tryptic mapping of haemoglobins by CE has been addressed in part by Ferranti *et al.* [14] who although reporting quantitative data failed to appreciate that electrokinetic loading has an inherent bias towards the more cationic species of a sample. Quantitation by conventional two-dimensional papier chromatography and electrophoresis cannot be performed "on-line" as it can with CE or HPLC. Further, CE has the advantage over gradient elution reversed-phase HPLC since it can be used with low UV wavelength detection where peptide bonds have a large molar absorptivity; the use of organic modifiers prohibits such detection in HPLC. The cost of the CE equipment and analysis compares very well with either HPLC or two-dimensional paper chromatography and electrophoresis. The same equipment can be used for other haematological analyses e.g. Hb variant red blood cell lysate [16] and whole globin separations [IS]. The main problem with this separation is one of reproducibility. In our experience great care must be taken to condition the capillary before analysis by washing it reproducibly, further, even very slight changes in!pH from buffer batch to batch can affect the resolution obtained which is quite critical with such a complex separation. Mass spectrometric detection is potentially ideal for peptide mapping since this can also give sequence information of the peptides [19], CE-MS has been compared with LC-MS for protein analysis [20] and is considered most effective for target compound analysis or in applications where only narrow mass ranges need to be scanned.

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