CHROM. 21 785

AUTOMATED EVALUATION OF TRYPTIC DIGEST FROM RECOMBINANT HUMAN GROWTH HORMONE USING ULTRAVIOLET SPECTRA AND NUMERIC PEAK INFORMATION[a]

HANS-JURGEN P. SIEVERT*

Hewlett-Packard Company, Route 41 and Starr Road, P.O. Box 900, Avondale, PA 19311 (U.S.A.)
and
SHIAW-LIN WU, ROSANNE CHLOUPEK and WILLIAM S. HANCOCK

Genentech, Incorporated, 460 Point San Bruno Boulevard, South San Francisco, CA 94083 (U.S.A.)

SUMMARY

UV spectra were successfully employed in identifying peptide fragments from a tryptic digest of recombinant-DNA-derived human growth hormone (r-hGH). It was possible to distinguish very similar peptides utilizing a digital comparison of the UV spectra. An automated procedure was developed to generate a calibration library for the tryptic digest of a reference standard. The calibration library was then evaluated for reproducibility and selectivity and found to provide superior performance in correctly identifying ambiguous peaks as compared to the use of a conventional calibration table.

Spectral match factors together with numerical information, derived from peak retention time, area and height, were used to arrive at a "peak score" descriptive of the similarity between standard and sample peaks. "Peak scores" could be combined to calculate a "sample score" indicative of overall similarity between an unknown and a standard. The scoring procedure was automated to generate a final report without operator intervention and successfully assigned appropriate scores to similar as well as dissimilar samples, *e.g.*, native and oxidized r-hGH.

INTRODUCTION

One method for the characterization of recombinant-DNA-derived proteins or peptides utilizes digestion with trypsin to obtain a number of characteristic peptide fragments which can be separated and quantified using high-performance liquid chromatography (HPLC)[1,2]. This "tryptic map" provides a key analytical technique that can be applied during the development stage as well as in the routine quality control of manufactured lots. However, the unambiguous identification of all peaks in the tryptic map is a laborious exercise which cannot be repeated on a day-to-day basis.

Thus, a typical lot release procedure relies on comparing replicate analyses of the tryp-tic map for the sample in question with those of well characterized reference material.

The comparison makes use of numerical peak data such as retention time, area and height. A major challenge in this context is that of assigning the correct identity to the chromatographic peaks obtained for each tryptic map. Careful control of the chromatographic equipment and separation conditions are required to reduce the variability of peak data to a minimum.

Peak assignment, however, becomes very difficult if more than one candidate peak is found in a given search window, a situation that is not uncommon with tryptic digests of larger proteins. Traditionally, the peak with the largest response in the window is considered to be the correct match. If additional chromatographic signals are available from parallel detection with the same or a second detector, the response of the standard under the different conditions of detection can also be used. With the advent of the diode array detector it becomes possible to obtain spectral information for each of the peaks in the tryptic map. This information has tremendous potential to facilitate peak identification by comparing the UV spectra of standard and unknown.

For a number of years, algorithms for the point by point numerical comparison of two spectra have been available and have been used successfully to distinguish between very similar compounds[3,4]. Utilizing one such algorithm, this study tried to determine whether UV spectra can be of use in the identification of the tryptic peptide fragments of recombinant human growth hormone (r-hGH). A critical question in this context was whether the spectra contain enough information to allow one to differentiate between compositionally very similar peptides, especially in the absence of aromatic amino acids.

Our study proceeded in four stages: (1) develop a procedure for the generation of a calibration library from reference standards; (2) determine the sensitivity and selectivity of this calibration library; (3) define an algorithm for calculating a peak score which reflects the confidence of correct identification; (4) combine individual peak scores to arrive at an overall similarity score to characterize a tryptic map.

EXPERIMENTAL

Chemicals and solvents

Acetonitrile (UV-grade, Burdick and Jackson), trifluoroacetic acid (spectro-grade, Pierce), water (purified by a Milli-Q system, Millipore) and trypsin (TPCK-treated, Sigma) were used. r-hGH (Protropin®) was produced as described previ-ously[5].

Oxidation of r-hGH

Samples were oxidized by adding 50 μl of chilled performic acid (9 parts 88% formic acid and 1 part 30% hydrogen peroxide) to 1 mg r-hGH and reacting the mixture for 1 h at 0°C.

Tryptic digest of r-hGH

Samples were digested in a buffer solution containing 100 mM sodium acetate, 10 mM Tris base and 1 mM calcium chloride at pH 8.3 at 37°C by addition of 1:100 trypsin (trypsin: r-hGH, by weight) at time zero and at 2 h. Samples were acidified after
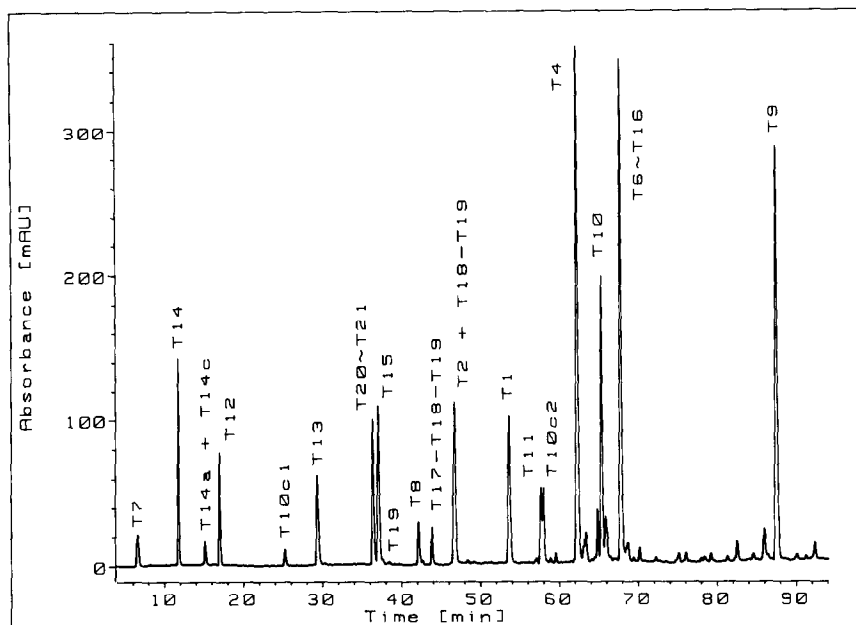
Fig. 1. Tryptic map of r-hGH separated with gradient I (TFA). A 100-μg sample was loaded in 0.2 ml of 0.1 *M* ammonium bicarbonate and separated using gradient system I as outlined in the Experimental section. The tryptic fragments as identified by FAB-MS are indicated in the figure, actual amino acid composition is as follows (c indicates a chymotrypsin-like cleavage; a indicates a pyroglutamic acid (q) containing residue; ~ denotes a disulfide bridge): T7 = EETQQK; T14 = QTYSK; T14a = qTYSK; T14c = QTY; T12 = LEDGSPR; T10c1 = SVFAN; T13 = TGQIFK; T20~T21 = IVQCR~SVEGSCGF; T15 = FDTNSHNDDALLK; T19 = VETFLR; T8 = SNLELLR; T17~T18~T19 = K~DMDK~VETFLR (incomplete digestion); T2 = LFDNAMLR; T18~T19 = DMDK~VETFLR (incomplete digestion); T1 = MFPTIPLSR; T11 = DLEEGIQTLMGR; T10c2 = SLVYGASDSNVYDLLK; T4 = LHQLAFDTYQEFEEAYIPK; T10 = SVFANSLVYGASDSNVYDLLK; T6~T16 = YSFLQNPQTSLCFSESIPTPSNR~. NYGLLYCFR; T9 = ISLLLIQSWLEPVQFLR.

a total of 4 h with 100 μl of phosphoric acid (pH < 3) per ml of sample and analyzed directly or stored for up to three days at 2–8°C. It had been determined elsewhere (unpublished results) that digestion of r-hGH was complete after 4 h.

High-performance liquid chromatography
        Separations were performed using a Hewlett-Packard 1090M HPLC system equipped with a DR5 ternary pumping system, an automated injection and sampling system, a heated column compartment and a diode array detector and controlled by an HP 79994A ChemStation.
        Two gradient systems were employed for the separation of the tryptic fragments. System I used trifluoroacetic acid (TFA) in water at 0.1% as solvent A, solvent B being 0.08% TFA in acetonitrile. The gradient was linear from 0 to 60% B between 0 and 120 min at a flow-rate of 1 ml/min with the oven temperature set at 40°C. System II utilized 50 m*M* sodium phosphate in water, pH 2.85, as solvent A, solvent B was acetonitrile. The gradient profile was linear from 0 to 40% B over 120 min at a flow-rate of 1 ml/min

with the oven temperature set to 40°C. For both gradient systems we used a 15 cm × 0.46 cm Nucleosil C_{18} reversed-phase column, particle size 5 μm, particle size 100 Å, packed by Alltech Assoc. Fig. 1 shows a typical chromatogram of a mixture of tryptic peptides derived from an r-hGH reference standard analyzed with the TFA gradient system.

Data processing

For all analyses, spectra were acquired at one-second intervals over the range from 200 to 350 nm. In addition, chromatographic signals were recorded at 220, 230, 254, 274, 280, and 292 nm with a reference wavelength of 350 nm in all cases. Rawdata were stored on magnetic media and were processed on the ChemStation using the built-in spectral library functions as well as additional evaluation software which was written for that purpose using a high-level command language available on the ChemStation.

Spectral matching

Numerical point by point comparison of two UV spectra is implemented on the ChemStation with the COMPARE command[6] and is illustrated in Fig. 2 where spectra for peptides T13 and T14 are compared (Fig. 2a). At each wavelength, absorbance
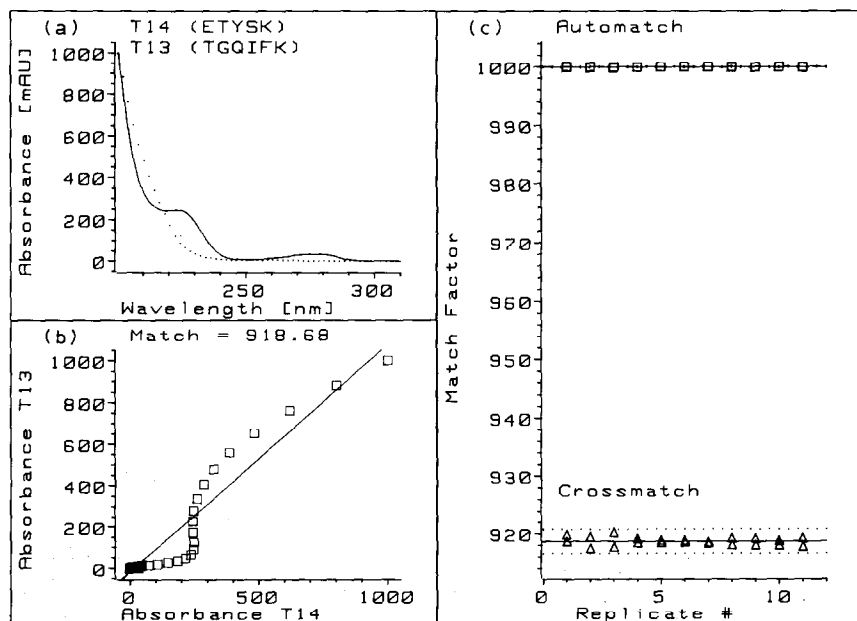

Fig. 2. Spectral match between tryptic peptides T14 and T13 as an example of moderate similarity. Part a shows the UV spectra for the two peptides (T13 shown as dotted line). Part b presents the distribution arising from plotting pairwise absorbance values for both peptides at identical wavelengths; the solid line is the linear least squares fit through the data. The square of the correlation coefficient multiplied by 1000 is defined as the match factor and is shown at the top of part b. Part c shows a comparison of the match factor for all of 11 spectra for the two peptides, either comparing each with its own average spectra (automatch) or with the average for the other peptide (crossmatch). The mean match factor is given as solid line in each case, the dotted lines indicate the upper and lower limits of ±3 standard deviations, respectively.

values for the two peptide spectra are plotted as abscissa and ordinate and a linear regression is applied to the resulting scatter plot (Fig. 2b). The square of the correlation coefficient, multiplied by 1000, is defined as the match factor for the two spectra. A value of 0 indicates no match at all, a value of 1000 would characterize a perfect match. The two peptides shown in Fig. 2a differ in the nature of the aromatic amino acid residue which is phenylalanine for T13 and tyrosine for T14. Their spectra are clearly different, even on visual comparison, and the match factor accordingly has a low value of 919. Thus, even though a match factor could be as low as 0, spectral dissimilarities are quite apparent to the naked eye once the match factor drops below 900.

    Fig. 3 illustrates how the match factor is affected when we compare T13 with T12, a peptide fragment which does not contain any aromatic amino acid at all (Fig. 3a). The spectra now are very similar and the match factor increases to 997 (Fig. 3b), approaching the value expected for identical spectra. Visual identification of the two compounds could at best be considered ambiguous. We will show later how the significance of a given match factor, even in close proximity of 1000, can be assessed in statistical terms.

RESULTS AND DISCUSSION

Spectral calibration library

    The first step towards characterizing the tryptic map of r-hGH was to compile a library of standard spectra for the various fragments in the map. For this purpose a reference standard was injected four times and analyzed with gradient systems
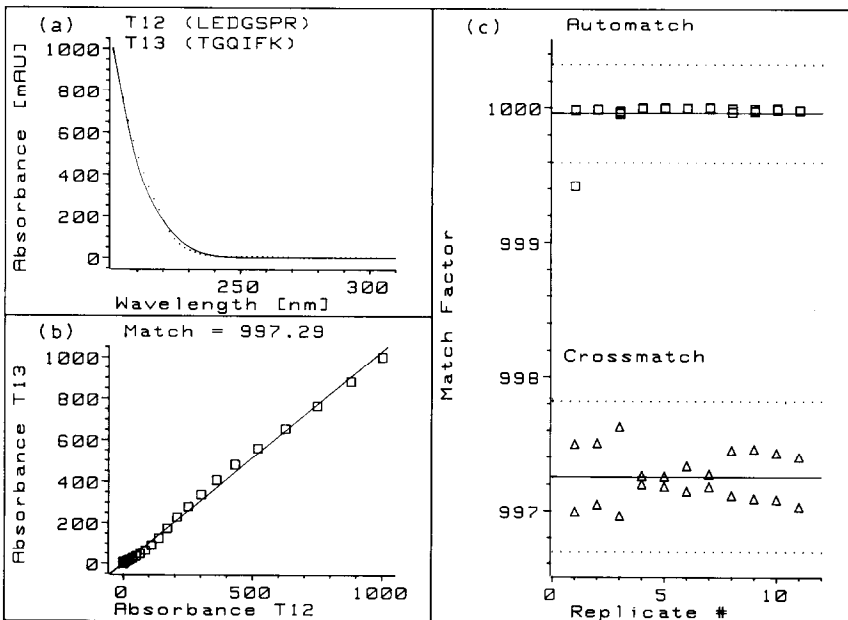

Fig. 3. Spectral match between tryptic peptides T12 and T13 as an example of strong similarity. The presentation of data is as explained in the legend to Fig. 2.

I (TFA based) and II (phosphate based). Each of the resulting data files was processed in the following fashion. After integration of the signal at 220 nm, apex spectra were identified for all integrated peaks. They were corrected for solvent background by subtracting a reference spectrum which was interpolated from two baseline spectra at either side of the peak. The resulting peak spectra were then stored into a library file which we referred to as a sample library since it contained all spectra characteristic of a given sample.

The two-point reference correction employed here is especially important in the case of gradient I, since TFA undergoes a significant change in spectral properties as the acetonitrile concentration is increased during the course of the gradient elution[7]. Fig. 4 illustrates how the uncorrected upslope and downslope spectra for fragment T9 differ significantly from the apex spectrum and each other (Fig. 4a). After baseline correction all three spectra match closely (Fig. 4b).

We next used a retention time window of ± 0.5 min centered on the apex of each peak from the first standard to find the spectrum with the best match from each of the other three standards. Those spectra that were common to all four standards were then averaged, normalized, smoothed, and transferred into a new spectral library file which we named the calibration library. For each peak of the tryptic map, this library file contains the UV spectrum and values for area, height, retention time, and scaling factor, all based on averages from the four standard runs.
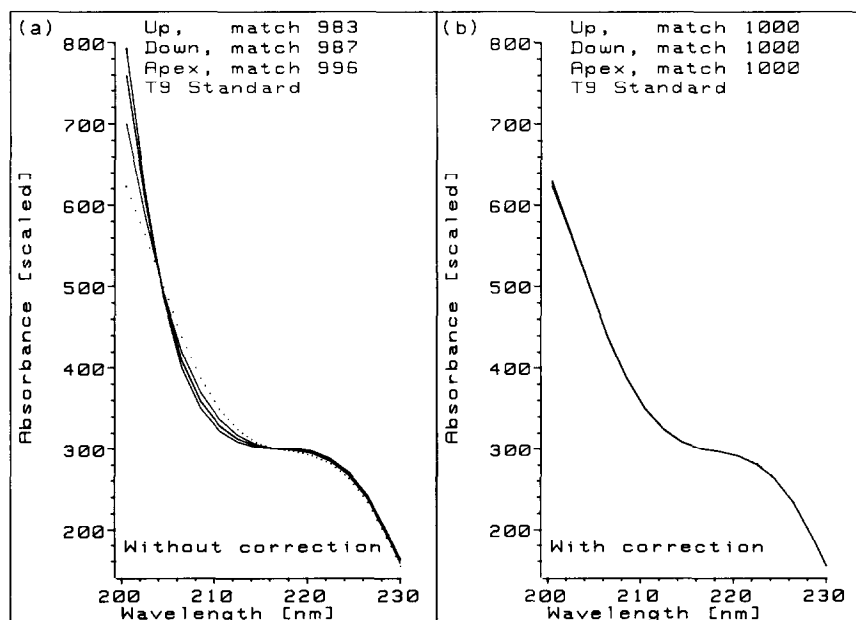

Fig. 4. Background correction for peak spectra as demonstrated for the tryptic peptide T9 separated with gradient I (TFA). Part a shows the comparison of uncorrected upslope, downslope and apex spectra for the T9 peak with a standard T9 spectrum (dotted line); match factors are given to indicate the degree of similarity. Part b presents the same spectra after background correction had been applied, match factors again refer to comparison with T9 standard.

From previous characterization studies the identity of the tryptic fragments had been determined by amino acid analysis and fast atom bombardment (FAB) mass spectrometry (MS)[1]. Library entries for peaks eluting prior to the first and after the last tryptic fragment as well as entries for peaks with area or height below one percent of total area or height were then removed. It had also been shown[1] that most of the minor peaks were not related to r-hGH but were nonspecific background, presumably derived from trypsin or due to other interferences like baseline noise or solvent impurities. The final calibration library for the TFA system contained 40 entries, 19 of which represented tryptic fragments of known identity; the phosphate library in its final form consisted of 31 entries. These two calibration libraries were used in all subsequent experiments.

Correlation of data from different standard runs relies heavily on good chromatographic reproducibility. In Fig. 5, chromatographic traces from four replicates analyzed with gradient II are overlaid to demonstrate that instrument performance is excellent even towards the end of the gradient. Statistical analysis of retention time variations showed the average standard deviation for all peaks incorporated into the calibration library to be 0.027 min (1.6 s) and 0.021 min (1.3 s) for gradient system I and II, respectively.

Reproducibility and selectivity of the calibration library

Two key properties of the match factor that determine the usefulness of the spectral data incorporated into the calibration library are reproducibility and
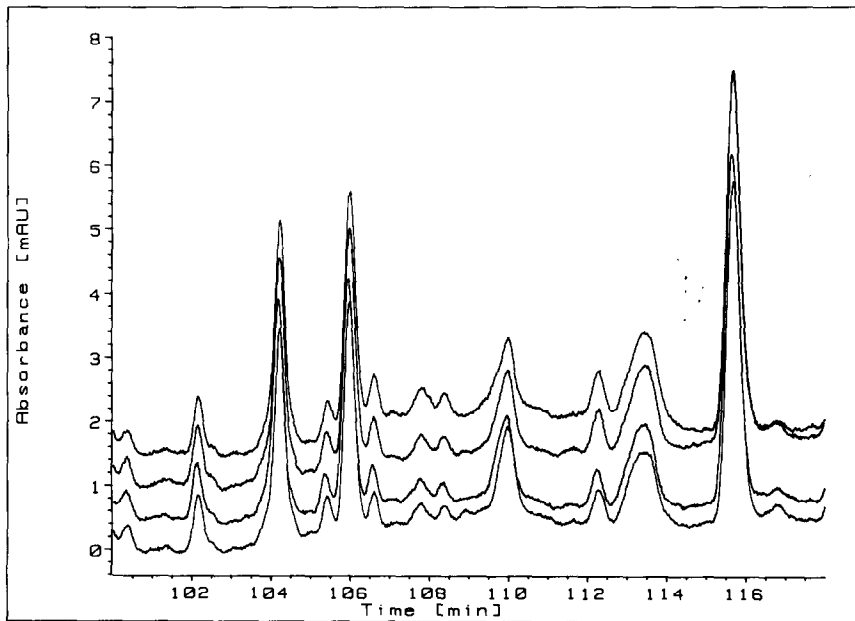

Fig. 5. Reproducibility of the tryptic map analyzed with gradient II (phosphate). A 100-µg sample was loaded in 0.2 ml of 0.1 M ammonium bicarbonate and separated using gradient system II as outlined in the Experimental section. The figure shows the superimposition of four replicate elution profiles. Some of the data have been shifted along the absorbance axis to provide greater clarity.

selectivity. We therefore decided to investigate these properties in a systematic fashion in order to obtain some quantitative guidelines. Results in this section were obtained using gradient I, since TFA, when employed as modifier, presents a greater challenge for LC detector and pump than does phosphate.

Reproducibility of the match factor determines the absolute limit for the similarity between any two spectra and thus defines the sensitivity of spectral matching. Two spectra can be considered different only when mean and standard deviation for the match between the two differ significantly from those obtained by repeatedly matching identical spectra. It is not sufficient to use a match factor cutoff as criterion for positive identification, additional statistical information is needed to determine the significance of a given match factor.

Spectra for T13 or T14 derived from eleven different injections were averaged to obtain a representative spectrum for each peptide. All individual spectra were then matched against their respective average (Fig. 2c, automatch; T13 ↔ T13av, T14 ↔ T14av) and the resulting distribution of match factors was compared with that obtained from matching individual T13 spectra against the average T14 spectrum and *vice versa* (Fig. 2c, crossmatch; T13 ↔ T14av, T14 ↔ T13av). It can be seen that the means for automatch and crossmatch are quite different, the match factor for the crossmatch of 918.6 is certainly a good indication of dissimilarity. More importantly, confidence intervals of three standard deviations above and below each mean as indicated in Fig. 2c do not overlap, but show a significant gap. Thus, we can distinguish T13 from T14 with a great degree of confidence.

Fig. 3c shows the corresponding plot of automatch and crossmatch for T13 and T12. These peptides are very similar in their spectral characteristics as can be seen by the mean crossmatch score of 997.25. Nonetheless, there is still a clear gap between the confidence intervals for automatch and crossmatch, indicating that it is possible to differentiate between compounds of extreme similarity. In statistical terms, if we apply Student's t-test for unequal variances to the data in Fig. 3c, we obtain a t-value of 57 and a probability of better than 99.99% that the mean values obtained for automatch and crossmatch are indeed different.

The t-test for the comparison of T13 and T14 (Fig. 2c) results in a t-value of 542 and a probability of 100.00% that the spectra are different. t-Values expressing the similarity among the four aliphatic peptides (T7, T8, T11, and T12) ranged from 13 to 133 which is sufficient for a statistically valid distinction. (For a population size of 11 a t-value of at least 6.2 is required to provide greater than 99.99% probability that two means are different.)

When we analyzed the reproducibility of match factors for the four standard runs using gradient I, we found that the mean match factor ranged from 998.76 to 1000.00 with standard deviations from less than 0.001 to 1.306. This indicated to us that we could employ very stringent match criteria for spectral identity. Since variability of the match factor increases as peak concentration decreases and since the relative concentrations of the tryptic fragments from r-hGH should be fairly constant, we decided to define individual match criteria for each entry in the calibration library rather than using a fixed match threshold. To be considered a positive match, an unknown spectrum had to have a match score above a threshold of three standard deviations below the mean match for a given standard. This gives us a 99.8% probability that only correct matches are assigned.

Our next concern was the selectivity of the calibration library, that is, its stability against false positive matches. To establish selectivity, each standard in the calibration library had to be matched against every entry from a typical sample library to determine the number of potential mismatches. A mismatch in this context was defined as a standard entry for which more than one match candidate was found with a match factor inside the confidence limits established earlier. Selectivity can be greatly enhanced by defining a retention time window around a given standard to limit the number of search candidates.

When we used a retention time window of $\pm$ 1 min, which is twice the window employed for later experiments, we found incorrect matches only for three standards. These mismatches were all minor peaks with peak heights between 3 and 6 mAU and did not correspond to any known tryptic fragments of r-hGH. With a $\pm$ 0.5 min window no mismatches were found at all. We concluded from this that, with selection of an appropriate retention time widow, the calibration library for r-hGH provides accurate identification of all fragments.

Traditional calibration procedures for peak identification based only on retention times resulted in mismatches for 5–8 standards inside a $\pm$0.5 min retention time window. When the window was increased to $\pm$1 min, nearly all standards exhibited mismatched peaks. Thus, the spectral information contained in our calibration library presents some clear advantages in reducing the potential for incorrect peak matching.

Definition and application of the peak score

Since chromatographic conditions are not always stable, resolution between adjacent peaks might change or additional peaks might appear in a tryptic map making it difficult to positively identify an unknown peak even when spectral matching is employed. However, in addition to peak spectra, other quantitative information is available for each peak and could be utilized to develop a procedure that would assign a numerical similarity score to each match between a standard and an unknown peak. Table I shows the variability of the different parameters available to construct this score. Based on the relative standard deviations, it is obvious that the greatest confidence can be placed in the match factor. Retention time information on one hand and peak area and height on the other hand exhibit deviations larger than those for the match factor by one and two orders of magnitude, respectively.

These findings are not surprising, if one considers that variability of area and height can be attributed not only to the chromatographic separation but also to the sample preparation, particularly the tryptic digestion. Retention time deviations, in turn, are primarily affected by variations in the chromatography. Assuming proper background correction, UV spectra should be completely invariant even under conditions that lead to noticeable fluctuations in retention times. The major cause for variability of the match factor would be detector noise, especially for fragments present at low concentrations.

Based on the statistical information in Table I, we can empirically define the following peak score (PS) which weights each parameter according to its variability:

$$PS = \{10 \times \text{Delta(MF)} + \text{Delta(RT)} + 1/10 \times [\text{Delta(AR)} + \text{Delta(HT)}]\}/11.2 \quad (1)$$

with

$$\text{Delta(MF)} = [\text{MF(Std)-MF(Unk)}]/[3 \times \text{sdev(MF)}], \text{MF(Std)} > \text{MF(Unk)} \qquad (2)$$

$$\text{Delta(MF)} = 0, \qquad\qquad\qquad\qquad\qquad \text{MF(Std)} \leqslant \text{MF(Unk)}$$

$$\text{Delta(RT)} = |\text{RT(Std)-RT(Unk)}|[3 \times \text{sdev(RT)}] \qquad\qquad\qquad (3)$$

$$\text{Delta(RA)} = |\text{RA(Std)-RA(Unk)}|/[3 \times \text{sdev(RA)}] \qquad\qquad\qquad (4)$$

$$\text{Delta(RH)} = |\text{RH(Std)-RH(Unk)}|/[3 \times \text{sdev(RH)}] \qquad\qquad\qquad (5)$$

Unk refers to the unknown, Std to the standard, and sdev indicates the standard deviation obtained for the four replicate standards. MF, RT, RA and RH are match factor, retention time, relative area and relative height, respectively; area and height values are expressed relative to a reference peak to eliminate concentration effects. To avoid unrealistically high delta values, we established minimum values for sdev of 0.1 (MF), 0.05 min (RT) and a 1% relative standard deviation for RA and RH.

   Eqn. 1 accounts for the fact that the spectral match is the most significant parameter for peak recognition and therefore is weighted most heavily. Even if all other parameters indicate a perfect match, a large deviation in the match factor

TABLE I

STANDARD DEVIATIONS FOR RETENTION TIME, AREA, HEIGHT, AND MATCH FACTOR OF TRYPTIC DIGESTS FROM r-hGH ANALYZED WITH TWO DIFFERENT CHROMATO-GRAPHY METHODS

	Gradient I (TFA)[a]			Gradient II (phosphate)[b]		
	Mean[c]	Low[d]	High[e]	Mean[c]	Low[d]	High[e]
Retention time						
Standard deviation (min)	0.027	0.007	0.174	0.021	0.004	0.041
Relative standard deviation (%)	0.136	0.008	1.882	0.075	0.007	0.594
Peak area[f]						
Standard deviation (mAU · s)	0.568	0.006	3.498	0.403	0.006	2.847
Relative standard deviation (%)	6.265	0.006	33.508	4.004	0.006	40.485
Peak height[f]						
Standard deviation (mAU)	0.501	0.006	5.476	0.464	0.000	2.682
Relative standard deviation (%)	3.281	0.006	16.425	3.109	0.000	41.555
Match factor						
Standard deviation	0.156	0.000	1.306	0.080	0.000	0.661
Relative standard deviation (%)	0.016	0.000	0.131	0.008	0.000	0.066

   [a] Standard deviations are based on a calibration library of 40 peaks.
   [b] Standard deviations are based on a calibration library of 31 peaks.
   [c] Overall mean for all peaks in the calibration library of standard deviations calculated for each individual peak from four replicate injections of r-hGH.
   [d] Minimum value for the standard or relative standard deviations as defined in c.
   [e] Maximum value for the standard or relative standard deviations as defined in c.
   [f] Peak area and peak height counts were normalized to fragment T10 as 100.

indicates that the peak in question has the wrong identity. The scaling factor of 11.2 is the sum of all weighting factors and normalizes the peak score to unit weight. The use of a one-sided delta value for the match factor in eqn. 2 reflects the fact that only match factors below the standard should be penalized.

By definition, a perfect peak score would be zero, a score of 1 will give us a 99.8% probability that we would not miss any positive matches but usually indicates rather marginal similarity between standard and unknown. Peak scores for all entries in the four sample libraries used to construct the calibration library ranged from 0.002 to 0.465 with an average score of 0.051. Because the score is open-ended, we somewhat arbitrarily decided that a score of 2 or larger indicated a totally mismatched peak. The probability that a positive match would result in a score of 2 is less than 0.0000002%.

Automated evaluation of digests using a sample score

At this point we had in our hands a quantitative procedure to describe how well a peak from a calibration library is matched by any given peak in an unknown sample. Our next step was therefore aimed towards developing a scoring procedure that describes the overall similarity between all the peaks in an unknown and a calibration sample. Such a sample score would make it possible to evaluate and score a tryptic digest in completely automated fashion requiring no operator intervention.

Our definition of the sample score (SS) is:

$$SS = \frac{1}{N} \left(\sum_{i=1}^{N} SS_i + 2 \times MP + EP \right) \tag{6}$$

where MP is the number of missed standard peaks, EP the number of extra peaks in the sample, and N the number of standard entries in the calibration library.

The sample score allows us to account for missed calibration peaks as well as for supernumerary peaks found in a sample. Furthermore, the score is normalized so as to be independent of the number of entries in the calibration library which becomes important if the library is modified. A peak score larger than 2 has previously been defined as a mismatch, therefore all peak scores are truncated to 2 so that missed and mismatched peaks have the same peak score. The penalty score of 1 for extra peaks is strictly empirical at this point, another possible approach could be to have the penalty reflect the size of the extra peak.

Even though a perfect sample score is easily defined as being exactly zero, it is more difficult to arrive at a criterion for what constitutes the limit between a passing and a failing score. Meaningful limits will have to be established through statistical analysis of typical sample scores for reference standards to account for variability due to different lots of growth hormone and trypsin as well as overall chromatographic variability.

Table II gives the sample scores for the four sample libraries (1A–D) used to construct the calibration library as well as for additional samples (2A–C and 3A–D) derived from the same reference standard but injected in different amounts. As expected, the calibration samples themselves, (1A–D), injected at 100 μg, show a very good score of 0.076 or less, with an average value of 0.050 indicative of the extreme similarity between all four replicates.

TABLE II

SIMILARITY BETWEEN REPLICATE SAMPLES OF TRYPSIC DIGESTS OF r-hGH ANALYZED WITH GRADIENT I (TFA)

Sample[a]	Amount (μg)	Individual sample scores[b]				Average sample score[b]
		A	B	C	D	
1	100	0.076	0.025	0.048	0.052	0.050
2	50	0.882	0.793	0.719	–	0.798
3	200	0.341	0.499	0.553	0.379	0.443

[a] All samples were tryptic digests of r-hGH reference material analyzed as described in the Experimental section.

[b] Sample score as defined in eqn. 6.

The increase in sample score for the 50-μg injections (2A–2C) to an average value of 0.798 is partly due to a drift in chromatographic conditions resulting in resolution changes for several peaks. The coeluting fragments T14a and T14c were separated into two peaks, each with a spectrum different from the composite spectrum contained in the calibration library. The partially resolved peak pair T11 and T10c2 (Fig. 1), on the other hand, was not separated at all and consequently neither fragment was identified. Furthermore, the fragment with the lowest concentration (T19) was not detected at this smaller sample size.

The 200-μg injections (3A–D) show an average score of 0.443 and thus fall between the 100- and the 50-μg samples. The increased sample score results from the same problematic peaks encountered with the 50-μg injection. In both the 50- and the 200-μg injections, the additional standard peaks which were missing were all small peaks of unknown identity. This would indicate that the significance of these unidentified peaks with respect to sample identity needs to be investigated in some more detail.

For the phosphate gradient system (gradient II), similar data are shown in Table III. Again, the four calibration samples (1A–D) exhibit very low scores of 0.064 and

TABLE III

SIMILARITY BETWEEN REPLICATE SAMPLES OF NATIVE AND OXIDIZED TRYPTIC DIGESTS OF r-hGH ANALYZED WITH GRADIENT II (PHOSPHATE)

Sample	Amount (μg)	Individual sample scores[a]				Average sample score[a]
		A	B	C	D	
1[b]	100	0.025	0.016	0.037	0.064	0.036
2[b]	100	0.671	–	–	–	0.671
3[c]	100	1.687	1.723	1.677	1.681	1.692

[a] Sample score as defined in eqn. 6.

[b] Sample was tryptic digest of r-hGH reference material analyzed as described in the Experimental section.

[c] Sample was tryptic digest of oxidized r-hGH reference material analyzed as described in the Experimental section.

less, with the average at 0.036. An additional sample (2), which also contained reference material, but was analyzed at a different time, shows a higher score of 0.671. This score is in the range of scores obtained for the 50- and 200-μg injections of reference material with gradient I. Closer inspection revealed that here, too, changes in peak resolution had an adverse effect on the sample score.

To provide some data on the kind of sample score obtained with a sample that is known to differ from the standard, we also analyzed samples of r-hGH which was oxidized prior to digestion with trypsin to simulate potential degradation pathways. As can be seen quite clearly (Table III, 3A–D), the average sample score of 1.692 lies significantly above the scores obtained for reference material and reflects the difference between oxidized and native r-hGH. Furthermore, reproducibility for the four samples is very good, indicative of the similarity among replicate injections of the oxidized samples.

To relate this abstract score to the more traditional visual method of evaluation, Fig. 6 shows a chromatogram for the oxidized r-hGH digest. Peaks that disappeared due to oxidation and those peaks that appear as new fragments and are not encountered in native r-hGH are clearly labeled. Although it is quite obvious, even to the casual observer, that the chromatogram in Fig. 6 differs considerably from the standard fragmentation pattern as indicated by the arrows, there are two clear advantages to the use of the sample score: (1) the whole evaluation procedure can be automated to obtain a final sample score without the need for operator intervention; (2) the scoring procedure is completely digital and therefore not subject to observer bias.
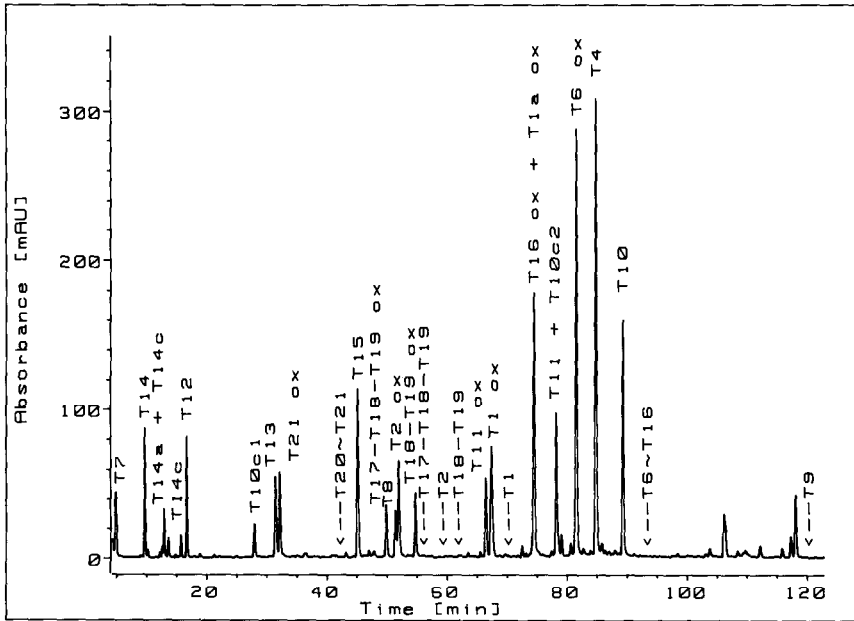

Fig. 6. Tryptic map for oxidized r-hGH analyzed with gradient II (phosphate). A 100-μg sample of oxidized r-hGH was loaded in 0.2 ml of 0.1 M ammonium bicarbonate and separated using gradient system II as outlined in the Experimental section. The elution position for the unoxidized peptides is indicated by arrows, oxidized peptides are denoted by "ox".

CONCLUSION

We believe that we have been able to show that, in spite of the overall similarity in their UV spectra, the individual peptides of the tryptic digest of r-hGH can be identified with a great deal of confidence. We developed a scoring procedure for tryptic digests that incorporates the cumulative similarity between the peaks in unknown and standard samples to assign a digital score to the match between the two.

At this point there are three major areas where further work is needed. The problem of mixed component spectra has to be addressed, possibly with the use of multi-component analysis. The significance of the small peaks of unknown identity, which might reflect lot to lot variability of either growth hormone or trypsin, requires further investigation. Finally, more data on the overall variability of the scoring procedure are needed to define the criteria that determine the cutoff between a pass or fail score.

Refinements in both the peak and the sample score could incorporate very specific knowledge about the expected behaviour of the r-hGH digest after chemical modification. Disappearance of peaks characteristic of oxidation, for instance, could be coupled to a search for the known oxidation products with a corresponding adjustment to the sample score. System suitability criteria like resolution between critical peak pairs could also be used to modify the sample score. For quality control applications, the peak score could be adjusted to place a greater weight on area and height deviations.

The methodology described here is by no means limited to r-hGH, tryptic digests from other proteins could be analyzed in a similar fashion, although the validation of selectivity for the calibration library has to be undertaken for each tryptic map. Peptide fragments, by nature of their rather non-descript and similar spectra, probably are more difficult to deal with than other chromatographic samples of comparable complexity but with more spectral variety. Tryptic maps might therefore be a good test case in the development of future expert systems for compound identification in complex samples.

REFERENCES

1 W. S. Hancock, E. Canova-Davis, R. Chloupek, S. Wu, I. P. Baldonado, J. E. Battersby, M. W. Spellman, L. J. Basa and J. A. Chakel, *Banbury Report 29: Therapeutic Peptides and Proteins: Assessing the New Technologies*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1988, p. 95.
2 W. S. Hancock, *Chromatogr. Forum*, 1 (1986) 57.
3 D. M. Demorest, J. C. Fetzer, I. S. Lurie, S. M. Carr and K. B. Chatson, *LC · GC, Mag. Liq. Gas Chromatogr.*, 5 (1986) 128.
4 D. H. Hill, T. R. Kelly and K. J. Langner, *Anal. Chem.*, 59 (1987) 350.
5 K. C. Olson, J. Fenno, N. Lin, R. N. Harkins, C. Snider, W. H. Kohr, M. J. Ross, D. Fodge, G. Prender and N. Stebbing, *Nature (London)*, 293 (1981) 408.
6 A. Drouen, *The COMPARE Command, Information Note*, Hewlett-Packard, Waldbronn, Publication Number 12-5952-3725, 1987.
7 G. Winkler, *LC · GC, Mag. Liq. Gas Chromatogr.*, 5 (1986) 1044.