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Proteolytic-Based Method for the Identification of Human Growth Hormone*

ABSTRACT: Human growth hormone (HGH) is a relatively small protein consisting of 191 amino acids and has an average mass of 22,125 amu. The forensic analysis of proteins such as HGH must meet the analytical sufficiency requirements for the laboratory and consists of a binary approach. A suspected sample is analyzed as the whole protein for retention time and mass determination using high performance liquid chromatography equipped with a photodiode array and liquid chromatography mass spectrometry. Further fragmentation of the protein using a proteolytic enzyme adds another dimension to the specificity of the analysis. Porcine trypsin digests proteins in a very predictable manner and yields peptide fragments of the original protein that can be used as a means for fingerprinting the larger biomolecule. *In silico*, or theoretical, digestion of HGH by trypsin yields 21 peptides ranging in size from 1 to 23 amino acids in length. The larger fragments containing higher numbers of amino acids give more specificity to identifying a protein based on a fragment produced by the digestion of trypsin. Herein, the analysis of HGH using a proteolytic approach is presented that meets the Scientific Working Group for the Analysis of Seized Drugs (SWGDRUG) recommendations for the identification of unknown substances.

KEYWORDS: forensic science, forensic drug analysis, human growth hormone, steroid, proteomics, liquid chromatography mass spectrometry, proteolytic

Recent publicity surrounding the purported use of performance-enhancing drugs such as steroids and human growth hormone (HGH) by professional athletes has focused on the need for more stringent anti-doping testing protocols and the ability to identify unusual substances in laboratories. HGH is often seized and submitted to forensic drug laboratories in conjunction with steroids. The analysis of steroids is relatively straightforward; however, most forensic laboratories are not accustomed to analyzing exhibits of HGH and qualitative methods are needed to perform these analyses. Addressing this issue will enable accurate and consistent reporting of HGH and provide valuable intelligence as to the frequency in which it is encountered by law enforcement personnel. HGH is considered a small protein, but it is still *c*. 50 times larger than regularly encountered drug substances. As routine analysis is not amenable to these large biomolecules, HGH cannot be identified using the instrumentation and methodologies typically utilized in a common forensic drug laboratory.

In a standard forensic drug analysis laboratory, gas chromatography-mass spectrometry (GC/MS) is the confirmatory instrument of choice. Most controlled substances of interest have molecular weights that are less than 400 amu and are easily vaporized for multi-component separation and isolation by gas chromatography. The isolated drug is then usually fragmented by electron impact ionization and the results are compared to a standard for identification. Proteins present a unique problem for a typical forensic drug analysis laboratory. The size of proteins (>1000 amu) and their thermal instability make them poor choices for analysis by GC/MS, but well suited to a liquid phase separation coupled with a mass spectrometer. Therefore, a biochemical, proteolytic-based high performance liquid chromatography (HPLC) and liquid chromatography-mass spectrometry (LC/MS) approach to identifying proteins is necessary for the analysis of HGH (1–5). One aspect of proteolytic analysis is analogous to using electron impact ionization in GC/MS. While energetic electrons are used to fragment gaseous molecules in GC/MS to yield a unique pattern, a specific enzyme can be used to cleave a protein at specific and reproducible locations to also yield a unique pattern.

Similar to controlled substances, the forensic analysis of HGH must meet the analytical sufficiency requirements for the laboratory. Based on the SWGDRUG guidelines, an analytical method was developed consisting of a binary approach as outlined in Fig. 1. A suspected sample is analyzed as the whole protein for retention time and mass determination using HPLC equipped with a photodiode array (PDA) and LC/MS. Further fragmentation of the protein using a proteolytic enzyme adds another dimension to the specificity of the analysis. Porcine trypsin is an enzyme that specifically cleaves proteins after the carboxyl side of lysine or arginine amino acids, unless followed by the amino acid proline. This enzyme digests proteins in a very predictable manner and yields peptide fragments of the original protein that can be used as a means for fingerprinting the larger biomolecule (5,6).

HGH is a relatively small protein consisting of 191 amino acids and has an average mass of 22,125 amu (as reported by Sigma-Aldrich). *In silico*, or theoretical, digestion of HGH by trypsin yields 21 peptide fragments ranging in size from 1 to 23 amino acids in length; see Table 1. Of these 21 peptides, 17 sequences are five amino acids or longer. The larger fragments containing higher numbers of amino acids can provide more specificity to identifying a protein. They can also be successfully fragmented further using MS^n technology to sequence the peptide for further identification of the whole protein.

This article presents the analysis of HGH using a proteolytic approach via digestion with porcine trypsin. The Drug Enforcement Administration laboratories do not routinely analyze body fluids such as blood or urine; the analysis described is intended for

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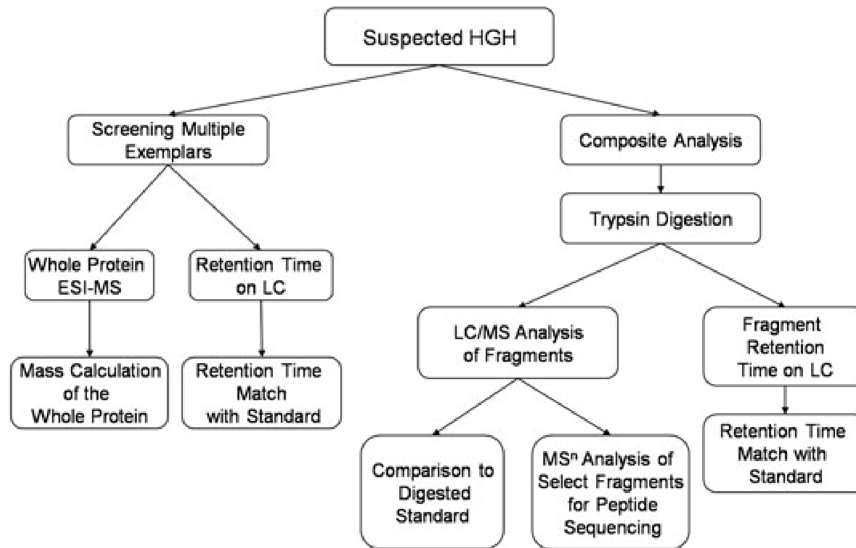

FIG. 1—*Analysis scheme. Multiple exemplars are composited after screening using whole protein mass calculation and LC retention time matching. Composites are digested using trypsin and the result is analyzed using LC and LC/MS.*

TABLE 1—In silico *digest of HGH.*

Peptide	Average Mass (H+)	Average Mass (2H+)	Sequence*
1	931.1		FPTIPLSR
2	980.1		LFDNAMLR
3	383.4		AHR
4	2343.5	1172.2	LHQLAFDTYQEFEEAYIPK
5	404.4		EQK
6	2617.8	1309.4 ($3H^+$, 873.2)	YSFLQNPQTSLCFSESIPTPSNR
7	762.7		EETQQK
8	844.9		SNLELLR
9	2056.4	1028.7	ISLLLIQSWLEPVQFLR
10	2263.5	1132.2	SVFANSLVYGASDSNVYDLLK
11	1362.5	681.7	DLEEGIQTLMGR
12	773.8		LEDGSPR
13	693.8		TGQIFK
14	626.6		QTYSK
15	1490.5	745.7	FDTNSHNDDALLK
16	1149.3		NYGLLYCFR
17	147.1		K
18	508.5		DMDK
19	764.8		VETFLR
20	618.7		IVQCR
21	785.8		SVEGSCGF

*Amino acid nomenclature:

A, alanine; R, arginine; N, asparagine; D, aspartic acid; C, cysteine; E, glutamic acid; Q, glutamine; G, glycine; H, histidine; I, isoleucine; L, leucine; K, lysine; M, methionine; F, phenylalanine; P, proline; S, serine; T, threonine; W, tryptophan; Y, tyrosine; V, valine.

Twenty-one (21) peptides are predicted from the HGH sequence provided by the supplier, Sigma-Aldrich. Peptides of shorter length are less significant because many proteins contain such sequences. However, the longer sequences are more specific and will enable definitive identification of HGH. Doubly charged fragments are also listed for those peptides that fall within the 600–2000 m/z range. One (1) triply charged fragment is given in the parentheses. The peptide numbering sequence is the convention used for numbering peaks in Fig. 6.

samples submitted prior to human consumption and matrices mimicking body fluids were not investigated. HPLC and LC/MS data provide identification of the whole protein and fragments based on retention time and molecular mass information. Tryptic digestion results are also compared with *in silico* digestion predictions.

Experimental

Protein Digestion

Digestion of an HGH standard obtained from Sigma (St. Louis, MO) was conducted using 2 μg of Modified Porcine Trypsin (Promega, Madison, WI, modified by methylation to minimize autolysis) in 245 μL of 50 mM NH_4HCO_3 with approximately 200 μg of HGH. A ratio of 150:1 (w/w), protein:trypsin, was targeted. Modified trypsin arrived from the supplier in 20 μg portions. This was resuspended in 50 μL of 50 mM acetic acid, also supplied by Promega. The resuspension was then divided equally into 10 × 5 μL aliquots containing 2 μg of trypsin each. The solution was heated using a thermostat set to 37°C and maintained at that temperature for *c*. 15 h. Proteolysis was quenched by cooling in a thermostatically controlled freezer at −40°C. The HGH standard obtained from Sigma was dissolved in water to reach a concentration of 1.5 mg/mL for whole protein analysis. Digestion was achieved using 300 μg of HGH with 2 μg of trypsin; digested samples were diluted with water to 1.5 mL total volume for analysis.

HPLC

HPLC analysis was conducted using an Agilent 1100 Series HPLC (Waldbronn, Germany) with PDA ultraviolet (UV) detection. Parameters for the analysis of the whole protein are listed below and were adapted from a method utilized by the U.S. Food and Drug Administration.

Column	Zorbax 300SB-C_3 Column (2.1 × 150 mm, 5 μm)
Mobile Phase	A = 0.05% formic acid, 0.01% trifluoroacetic acid (TFA) in water
	B = 0.05% formic acid, 0.01% trifluoroacetic acid (TFA) in acetonitrile
	90% A: 10% B, hold for 1 min
	Ramp to 28% A: 72% B over 11 min
Temperature	30°C
Flow rate	500 μL/min
Wavelength	210 nm
Injection volume	25 μL
Injection solvent	Water

Parameters are listed below for the HPLC analysis of HGH digested with porcine trypsin.

Column	Phenomenex Luna C_{18} (3.0 × 150 mm, 3 μm)
Mobile phase	A = 0.07% TFA in water
	B = 0.07% TFA in acetonitrile
	95% A: 5% B
	Ramp to 80% A: 20% B for 20 min
	Ramp to 75% A: 25% B for 20 min
	Ramp to 50% A: 50% B for 25 min and hold
Temperature	30°C
Flow rate	700 μL/min
Wavelength	210 nm
Injection volume	100 μL
Injection solvent	Water

LC/MS

A ThermoFinnigan Scientific LCQ Advantage Max ion trap mass spectrometer (San Jose, CA) equipped with a ThermoFinnigan Surveyor HPLC was used in the positive electrospray ionization (ESI) mode. Instrumental parameters for the tryptic digest analysis are listed below. Whole protein analysis was infused into the mass spectrometer off-column using a syringe and syringe pump (5 μL/min) coupled with a co-flow of 100 μL/min 0.1% trifluroacetic acid in H_2O.

Column	Phenomenex Luna C_{18} 100Å (2.0 × 30 mm, 3 μm)
Mobile phase	A = 0.1% TFA in water
	B = 0.1% TFA in acetonitrile
	100% A hold for 8 min
	Ramp to 80% A: 20% B for 8 min
	Ramp to 75% A: 25% B for 10 min
	Ramp to 50% A: 50% B for 4 min
	Ramp to 100% B for 4 min and hold for 3 min
	Re-equilibration time of 10 min at 100% A
Temperature	Column at 30°C, sample tray at 4°C
Flow rate	100 μL/min
Injection volume	20 μL
Injection solvent	Water
Ionization mode	Positive ion mode, electrospray
Mass analyzer	Ion trap mass spectrometer
Acquisition mode	Full-MS scan
Scan parameters	600–2000 m/z
MS^2 scan parameters	Fragmentation = 35.0% normalized collision energy
	Injection time = 50 msec using Automatic Gain Control
	Isolation window = 1.0 m/z
	Activation time = 30 msec

Discussion

Whole protein analysis was conducted using a two-step approach. The first step utilizes liquid chromatography with UV detection to screen the sample for the presence of multiple proteins. A comparison of retention times to a known standard can be used

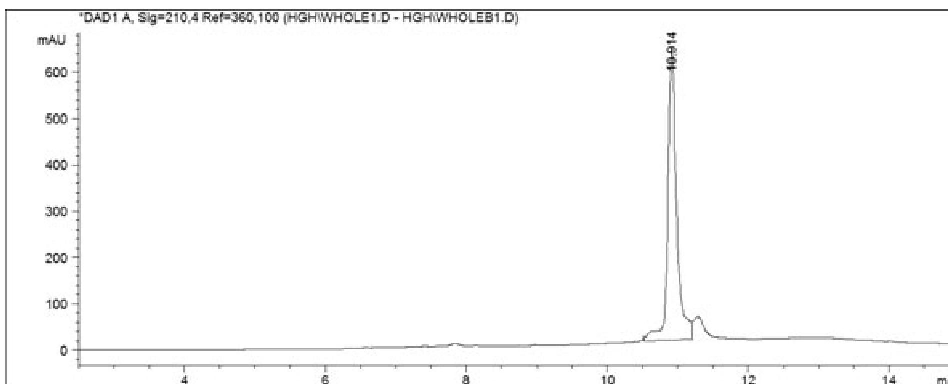

FIG. 2—*Whole protein chromatogram. A water blank was subtracted from the baseline.*

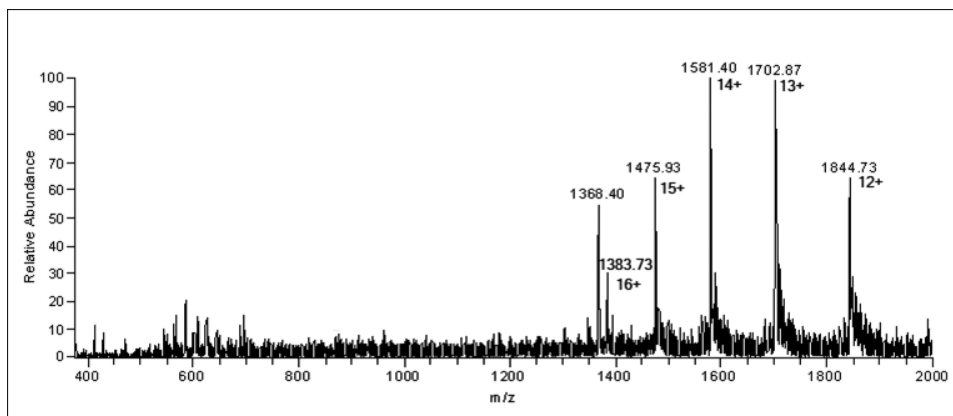

FIG. 3—*Mass spectrum of whole HGH. Due to the large size of HGH (22,125 amu), multiple charges are required so as to analyze the spectrum in a 1000–2000 m/z range. The peak at 1844 m/z is HGH with 12 protons, with each subsequent peak separated by a single charge (i.e., the peak at 1702 m/z sustains 13 protons and has a lower effective m/z value).*

for presumptive testing. An example of such a chromatogram is illustrated in Fig. 2. This screening technique can be coupled to a whole protein analysis using an LC/MS to provide a simple method for testing multiple exemplars before compositing a sample.

For most illicit drugs, a mass range of 50–500 m/z is sufficient to analyze the drug of interest. Although this range can be extended to 2000 m/z for most quadrupole mass filters, this mass window with traditional ionization techniques is still not sufficient to analyze even small proteins such as HGH given the mass of 22,125 amu. The development of ESI has allowed for the facile analysis of large biomolecules using common mass analyzers (7). The ESI process can result in a large molecule with multiple charging, reducing the observable mass-to-charge ratio (m/z) into the range where a typical quadrupole mass spectrometer can filter the mass. For example, a protein with a mass of 22,125 that sustains 15 protons will be detected at a m/z value of 1476. Figure 3 illustrates the charge state distribution obtained during ESI analysis of the intact HGH protein. Each peak observed corresponds to a different charge state of the protein, defined as $(M+nH^+)^{n+}$, where n is the number of charges (protons) attached. As observed, the multiple charging of the protein allows for its detection within the 1000–2000 m/z mass range window. For low resolution mass spectrometers, the m/z difference between adjacent charge states can be used to calculate the experimental molecular weight of the protein. This information can also be obtained via deconvolution of the charge state distribution or by using available software designed to assist in the sequencing of proteins, such as Bioworks (8). This

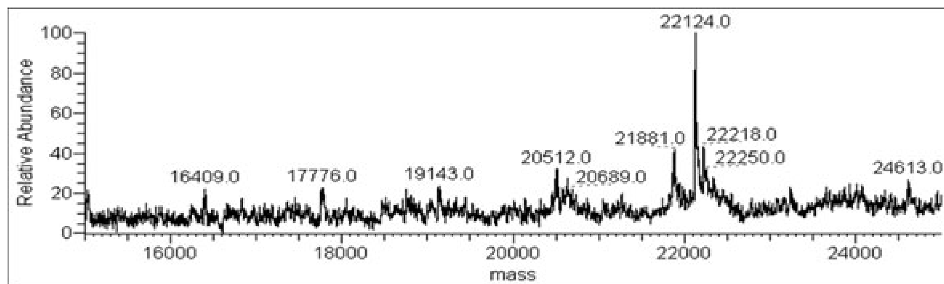

FIG. 4—*Bioworks software available from ThermoFinnigan allows for the deconvolution of the multicharged envelope. The mathematic calculation yields a deconvoluted mass of 22,124 amu which agrees very closely to the value given by the supplier of 22,125 amu; this correlates to a % error of only 0.004%.*

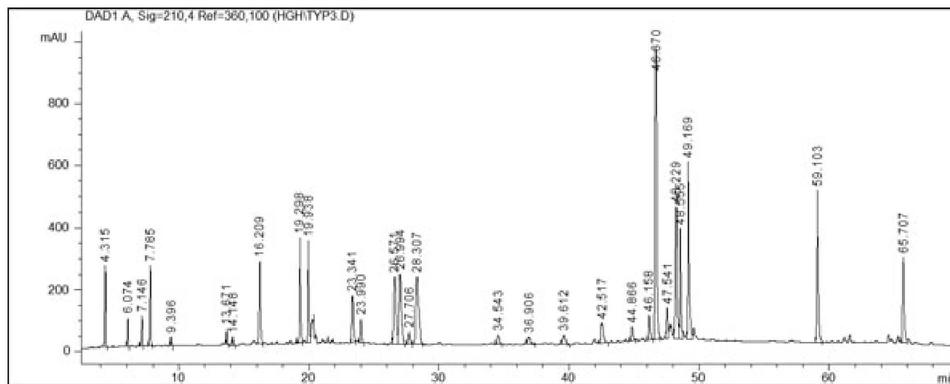

FIG. 5—*Trypsin digest HPLC chromatogram. Retention time matching for a drug of interest typically yields only a single peak. Using a proteolytic enzyme, a protein such as HGH is fragmented in many locations to yield numerous peaks for retention time matching.*

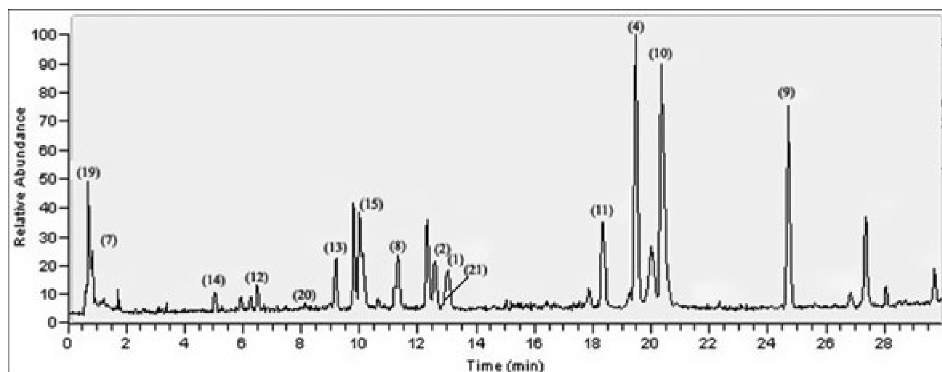

FIG. 6—*The total ion chromatogram (TIC) for trypsin digested human growth hormone using the LC/MS. The numbers on corresponding peaks are associated with the peptide number given in Table 1. Fragment 21 co-elutes with fragment 1 and appears as a slight shoulder on the peak.*

TABLE 2—*B and Y ions predicted for the fragmentation of peptide #4.*

Peptide 4	LHQLAFDTYQEFEEAYIPK		
Amino Acid	M+H	B	Y
L	114.1	114.1	2343.5
H	252.3	251.3	2230.4
Q	380.4	379.4	2093.2
L	493.6	492.5	*1965.1*
A	564.6	563.6	*1851.9*
F	711.8	*710.8*	*1780.9*
D	826.9	825.9	*1633.7*
T	928	927	*1518.6*
Y	1091.2	*1090.2*	*1417.5*
Q	1219.3	1218.3	*1254.3*
E	1348.4	1347.4	*1126.2*
F	1495.6	1494.6	*997.1*
E	1624.7	1623.7	849.9
E	1753.8	*1752.8*	720.8
A	1824.9	1823.9	591.7
Y	1988.1	*1987.1*	520.6
I	2101.2	2100.2	357.4
P	2198.4	2197.3	244.3
K	2326.5	*2325.5*	147.2

The masses *italicized* are clearly observed in the experimental data. B ions arise from the fragmentation of the peptide sequence where the charge remains with the N-terminus side of the peptide. Y ions arise from the charge retention on the C-terminus side. As the parent mass (1172 m/z) was doubly charged, resulting ions from the fragmentation can have a higher m/z if they are only singly charged.

is illustrated in Fig. 4 (BioworksBrowser 3.1 SR1 was used for the calculation), where deconvolution results in a peak at 22124 amu, which agrees with the molecular mass of HGH as indicated by Sigma-Aldrich. Comparison of the calculated mass of the HGH standard with the value reported by Sigma-Aldrich agrees to within 0.004%. As a protein gains higher levels of multicharging, the separation of isotopic contributions by the mass spectrometer cannot be reached without extraordinary resolution. As a result, the average molecular weight is used and this has an effect on the calculated value of a protein. This second test confirming the mass of the protein is sufficient to composite multiple exemplars for more in-depth analysis.

The second part of the binary analytical approach subjects composited samples to digestion using trypsin. The specificity of the enzyme allows for the facile comparison of an unknown sample to a standard using a liquid chromatography method. This comparison is far more specific than a typical presumptive test of comparing a whole protein unknown to a standard. For instance, the retention time of an illicit cocaine sample can be compared to the retention time for an authenticated cocaine standard. However, this comparison only utilizes a single peak for the presumptive test. Comparing a sample of human growth hormone that has been digested using trypsin will lead to numerous peaks for comparison that is analogous to a fragmentation pattern observed from an electron impact ionization mass spectrum. Figure 5 illustrates the complexity of the digested HGH and the specificity of using a digested protein for comparative analysis.

An even more specific method for identifying the fragments resulting from a trypsin digestion is to use LC/MS to determine the mass of the fragments created. While digestion of the protein and comparison of the fragments to a trypsin digestion of a known standard using HPLC indicates the presence of HGH in illicit samples, an additional step can be utilized to confirm the presence of HGH. An LC/MS method can be employed to both separate the digested fragments and to determine the mass-to-charge ratio of each. The mass of each fragment can also be compared to the values obtained through *in silico* digestion to further confirm the presence of HGH. In Fig. 6, the peaks are labeled

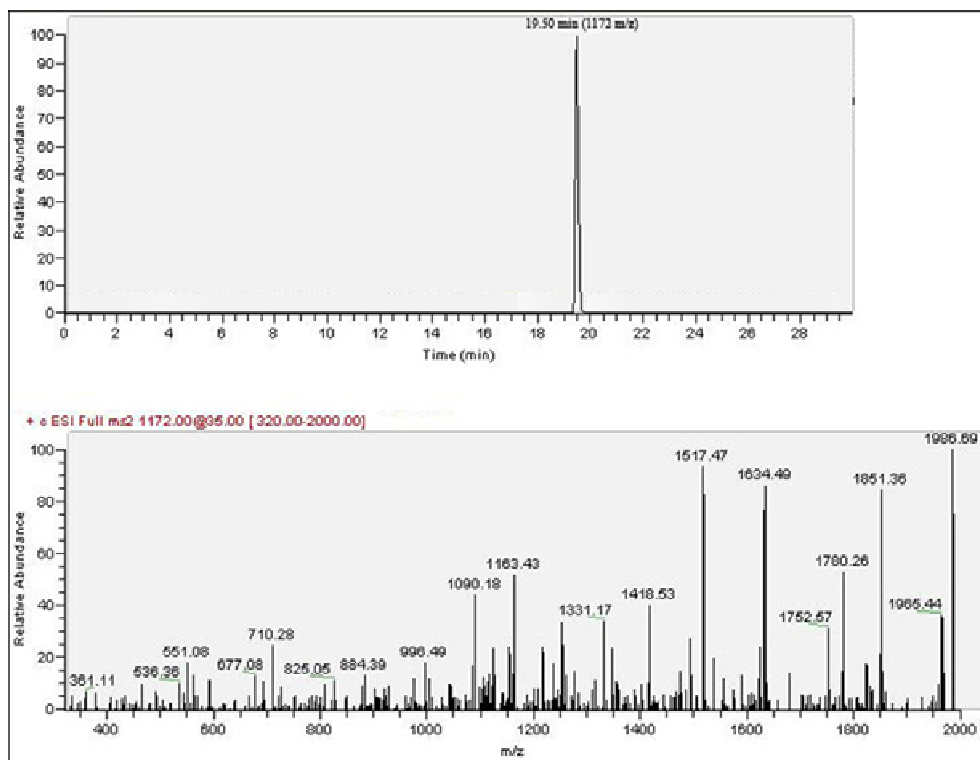

FIG. 7—*Above is the extracted ion chromatogram (EIC) for peptide #4. The peak at 19.50 min corresponds to the doubly charged peptide* $(M + 2H^{+})^{2+}$ *detected at m/z 1172. The bottom image is the* MS^2 *mass spectrum of 1172 m/z using 35.0% normalized energy for fragmentation.*

with the corresponding fragment generated by the *in silico* digestion. Of the 21 peptide fragments expected, 17 are in the mass range of 600–2000 m/z. Analysis using LC/MS identified 15 of 17 peptide masses expected which correlates to 82% coverage of the protein.

Additionally, further fragmentation of a specific peptide sequence using MS^n technology and comparison to the similarly analyzed fragment from a HGH standard can increase specificity. For example, peptide #4 was experimentally fragmented to yield a secondary mass spectrum that also correlates nicely with expected theoretical mass fragments predicted by Bioworks software. Table 2 lists Bioworks-predicted masses arising from the fragmentation of peptide #4. Figure 7 is the experimental data derived from the fragmentation of peptide #4. A strong correlation between the predicted masses and the experimentally obtained fragmentation provide further evidence that a proteolytic-based approach for the analysis of HGH is a suitable choice.

Conclusion

A method for the forensic analysis of HGH has been developed using HPLC and LC/MS; using a PDA in-line with the LC/MS can minimize the time necessary for analysis. The study of HGH is ongoing. Additional studies will compare the tryptic digests of several growth hormones. Preliminary studies of porcine growth hormone indicate that the whole protein mass and the trypsin digest profiles are dissimilar to HGH and are easily distinguished from one another. Additional work will involve investigating the identification of HGH in the presence of other proteins, as well as developing a facile method for the quantitation of mixtures.

Acknowledgments

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