In: Donike, H. Geyer, A. Gotzmann, U. Mareck-Engelke, S. Rauth (eds.)
Recent Advances in Doping Analysis (1). Sport und Buch Strauß, Köln 1994

L. D. Bowers and K. Fregien

HPLC/MS Confirmation of Peptide Hormones in Urine: An Evaluation of Limit of Detection

Indiana University Sports Medicine Drug Identification Laboratory, Indianapolis, IN, USA

Introduction

The detection of the use of peptide hormones such as human growth hormone (hGH), human chorionic gonadotrophin (hCG), adrenocorticotrophic hormone (ACTH), follicle stimulating hormone (FSH) and leutinizing hormone (LH) as performance enhancing agents in sport has been hampered by the lack of a definitive confirmation technique. Since the hormones are present in normal urine, the test must be quantitatively accurate as well as contain information content to verify the identity of the hormone. Although antibodies with differing epitope specificities are now available, complete characterization and calibration in the urine matrix has not been reported. In addition, monitoring of some peptide hormones, such as hGH, might be best accomplished through measurement of an induced peptide, such as insulin-like growth factor I (IGF-1) or somatomedin C.

Recent advances in mass spectrometry have allowed the quantitative analysis and sequencing of peptides and proteins of increasing molecular mass. Electrospray or its pneumatically-assisted derivative, Ionspray®, have been used for quantifying peptides in the attamole range. In addition, we have obtained structural identification on a few pmoles of the metabolites of the cyclic undecapeptide Cyclosporine. The present study investigated whether (1) the limit of detection using the Ionspray HPLC/MS interface was sufficiently low to allow confirmation of the peptide hormones at concentrations observed in urine; (2) the chemical and physical parameters of the interface could provide sufficiently precise ion ratios to support identification; and (3) what criteria will need to be established to support identification of the hormones.

Materials and Methods

A Sciex API III MS/MS equipped with an Ionspray interface was used throughout these experiments. The mass range of the quadrupole system was 10 - 2000 daltons. An HPLC system consisted of a Beckman Model 126 solvent delivery system, a Lee ViscoJet microliter

mixing chamber, a Rheodyne 7185 microbore injection valve, and a Keystone 1x150 mm DeltaBond C-18 column. The entire effluent flow of 50 µL/min was directed to the MS. Gradient elution was used throughout, with Solvent A consisting of 0.1% trifluoroacetic acid and Solvent B consisting of 0.1% trifluoroacetic acid in acetonitrile. Most of the chromatograms shown were run 90/10 A/B to 40/60 A/B over 20 minutes.

The peptide hormones studied were myoglobin, ACTH, LH, FSH, hCG, and hGH (Sigma), recombinant IGF-1 (Bachem), recombinant hGH (Lilly), and recombinant Methionine-hGH (Genentech). The latter two peptides were pharmaceutical preparations. The peptides were used without further purification or characterization.

Results

The performance capabilities of the HPLC/MS system for myoglobin are well known, and in fact myoglobin is used by many manufacturers as a system check at installation. The spectrum obtained from myoglobin and the detection capabilities of the HPLC/MS system are shown (Figure 1). Unfortunately, the detection characteristics of the Ionspray HPLC/MS system can depend rather strongly on the compound. Thus we studied the detection limits of a number of banned peptide hormones. Similar multiply charged ions were observed for ACTH, IGF-1 (Figure 2), and hGH (Figure 3). Note that as the molecular size of the peptide decreases, the number of charges observed decrease. In the spectrum of IGF-1, for example, only ions with 6, 5, and 4 charges are observed. Ions with less than four charges exceed the mass range of the instrument (2000 daltons). The spectrum of hGH is more complex due to the larger number of amino acids and therefore the charge distribution. The power of the technique is clear from this figure. Although there is a single additional amino acid in Genentech's Protropin product, the mass difference (1%) is clearly evident from the mass spectrum of the pure compounds. Thus Protropin could be clearly distinguished from native hGH.

We were unable to obtain reliable spectra for hCG, erythropoeitin (EPO), FSH, and LH. The reason for this problem was the lack of purity of peptide standards available from Sigma. The salts and contaminating peptides essentially undetectable by standard purity determination were sufficient to suppress ionization of the peptides of interest and to interfere in collection of an accurate spectrum. Studies with intact hCG, β-hCG, and β-core fragment purified with immunoaffinity columns and reversed phase HPLC are in progress.

In: Donike, H. Geyer, A. Gotzmann, U. Mareck-Engelke, S. Rauth (eds.)
Recent Advances in Doping Analysis (1). Sport und Buch Strauß, Köln 1994

Although we have demostrated the ability of HPLC/MS to accurately identify banned peptide hormones on the basis of their HPLC retention times and mass spectra-derived mass, determination of the peptides for amounts commonly found in urine presents a more difficult problem. We chose to monitor the three ions observed for IGF-1 (m/z 1297.5, 1557.5, and 1946) in the selected ion monitoring (SIM) mode and analyzed a series of aqueous standards over the concentration range 640 nmoles/L to 640 µmoles/L. Good linearity was observed over the entire three orders of magnitude in concentration. Injection of 50 fmol of IGF-1 onto the HPLC column gave a signal-to-noise ratio of 25-to-1 for the chromatographic peak (Figure 4).

Discussion

Electrospray and atmospheric pressure ionization of peptides, proteins, and oligonucleotides has been a major advance in the biological application of mass spectrometry[1-2]. The initial implementation of electrospray was such that only very low liquid flow rates could be used. The addition of pneumatic (Ionspray™) or thermal assist to the liquid nebulization process has extended the liquid flow rate compatibility to several hundred µL/min. The mechanism of ion production in Ionspray is not completely understood. Nevertheless, several general observations can be made. For macromolecules, ionization gives rise to a number of charged states, perhaps reflective of the state in solution. Since the mass spectrometer actually measures the mass-to-charge (m/z) ratio, this ionization process gives rise to a number of m/z ions. Since charge is integral and all ions originate from the same mass, it is possible to calculate an accurate mass of the original macromolecule from the ion pattern[3].

In addition to the information content of electrospray mass spectra, the technique has excellent detection limits. We have been able to detect 100 fmole of the cyclic peptide Cyclosporine[4] in tissue and 50 pg/mL of the cyclic macrolide rapamycin[5] in blood with better than 10% precision using HPLC/MS and HPLC/MS/MS. These compounds yield singly charged ions, but some observations can be made relative to the quantitative analysis of larger peptides and proteins. Larger proteins seem to have lower ionization yields, and thus there is a limit to the mass of the protein that can be detected. Second, ion production is quite compound specific. Thus, although one peptide species may be detectable in the fmole range, another may require nmole concentrations before it can be detected. This will require that each peptide be characterized individually. Finally, production of more ions of different m/z ratio may not be beneficial. If the same number of moles of charge is distributed over a larger number of ions, the detection limit of the peptide may be higher than the same charge distributed over fewer ions.

We were able to obtain spectra consistent with the molecular mass of the banned peptides ACTH and hGH, and for a related peptide, IGF-1. We were unable to obtain spectra for hCG, LH, FSH, and EPO. Our problem with impure peptide "standards" illustrates a significant problem in the investigation of HPLC/MS for peptide determination. Frequently the purity considered "state-of-the-art" in biochemical or physiological research can be shown to be multiple species by mass spectroscopy. An alternative is to obtain proteins derived from molecular biological studies, although in this case variations in post-translational modifications will have to be carefully monitored. It should be noted that microheterogeneity due to carbohydrate substitution yields a specific pattern with HPLC/MS and thus can be readily detected.

Criteria for identification of molecules based on the Ionspray interface will need to be determined. Unlike the unimolecular fragmentation reactions associated with odd-electron ions formed in electronic ionization GC/MS, the multiple charged species derived from electrospray are formed by multimolecular reaction dynamics near atmospheric pressure. It is unclear how quantitatively and precisely this process can be controlled on a continuing basis. The difference in ratio between the ions in Figure 4 relative to Figure 2 is in part due to an optimized SIM collection in the former versus 0.5 Da steps in the latter scan. Nevertheless, the reproducibility of three ion ratios at 50 fmol exceeds the 20% commonly accepted in GC/MS. Is the presence of three ions of the correct mass at the correct retention time sufficient to confirm the presence of a peptide? How many ions from an ion profile are necessary to identify a peptide? Does the use of immunoaffinity purification of the peptide from urine relax any of the above criteria?

Finally, a major consideration in the present study was to document sufficiently low limits of detection to measure peptide hormones in urine. We chose IGF-1 as a model system since it is induced by hGH, it has a half-life of several days, and we were able to obtain pure recombinant IGF-1 as a standard. We have demonstrated a limit of detection of about 10 fmol for IGF-1 with a 1000-fold linear dynamic range. The reported "normal range" for IGF-1 is 66-80 pg/mg creatinine in urine. Making the conversion to fmole/mL and assuming that 100% of the IGF-1 can be extracted from 10 mL of urine via immunoaffinity solid phase extraction, the 100 fmol of IGF-1 could be readily detected with the three ion SIM HPLC/MS conditions described here. We conclude, therefore, that additional studies on this technique are warranted.

In: Donike, H. Geyer, A. Gotzmann, U. Mareck-Engelke, S. Rauth (eds.)
Recent Advances in Doping Analysis (1). Sport und Buch Strauß, Köln 1994

References

1. Fenn JB, Mann M, Meng CK, Wong SF. Electrospray ionization - principles and practice. *Mass Spectrom Reviews* 1990; 9: 37-70.

2. Smith RD, Loo JA, Edmonds CG, Baringa CJ, Udseth HR. New developments in biochemical mass spectrometry: Electrospray ionization. *Anal Chem* 1990; 62: 882-99.

3. Mann M, Meng CK, Fenn JB. Interpretting mass spectra of multiply charged ions. *Anal Chem* 1989; 61: 1702-8.

4. Whitman DA, Abbott V, Fregien, K, Bowers LD. Recent advances in HPLC/MS and HPLC/MS/MS: Detection of Cyclosporine and metabolites in kidney and liver tissue. *Ther Drug Monit* 1993; (in press).

5. Bowers LD, Fregien K. Measurement of rapamycin in whole blood by HPLC/MS/MS. *Biol Mass Spec* 1993; (submitted).

In: Donike, H. Geyer, A. Gotzmann, U. Mareck-Engelke, S. Rauth (eds.)
Recent Advances in Doping Analysis (1). Sport und Buch Strauß, Köln 1994

Figure Captions

Figure 1. A mass spectrum of 64 pmoles of myoglobin showing a typical gaussian distribution of multiply-charged species (top). The ion at m/z 943 has 18 charges while the ion at m/z 1884.5 has 9 charges. The HPLC/MS trace (bottom) shows detection of 6.4 pmoles of myoglobin using four ions from the mass spectrum in the selected ion monitoring (SIM) mode.

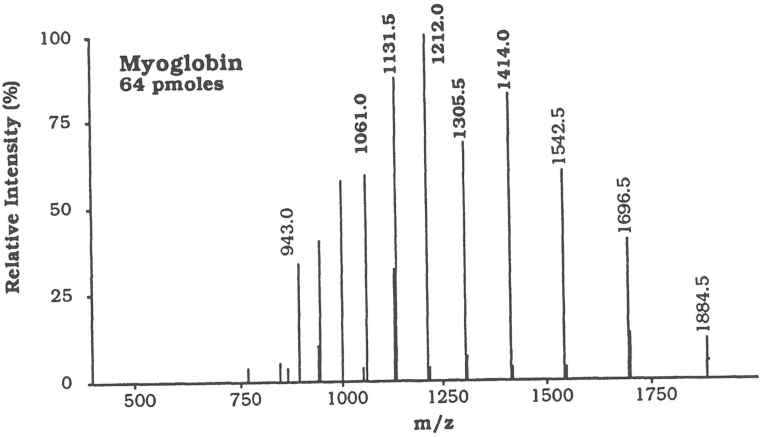
Figure 2. Mass spectra of ACTH (top) and IGF-1 (bottom). The mass derived from the observed ions is given below the peptide name and indicates the mean and standard deviation of the calculated mass of the intact molecule.

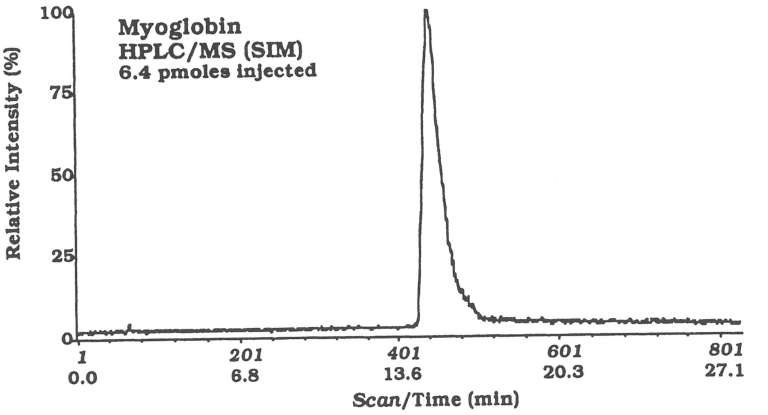
Figure 3. Mass spectra of pharmaceutical preparations of hGH (Humatrope (Lilly)) and methionine-hGH (Protropin (Genentech). The addition of a methionine residue in a molecule containing 191 amino acids is sufficient to allow mass descrimination. Note that all of the ions are shifted significantly in m/z, thus allowing identification based on a subset of the entire spectrum.

Figure 4. Selected ion monitoring (SIM) profile of 50 fmol IGF-1 injected onto the HPLC/MS system. The chromatographic trace (top) indicates that the limit of detection of 20 fmol is feasible. The three ions used for the SIM show the ratio obtained at 50 fmol (bottom).

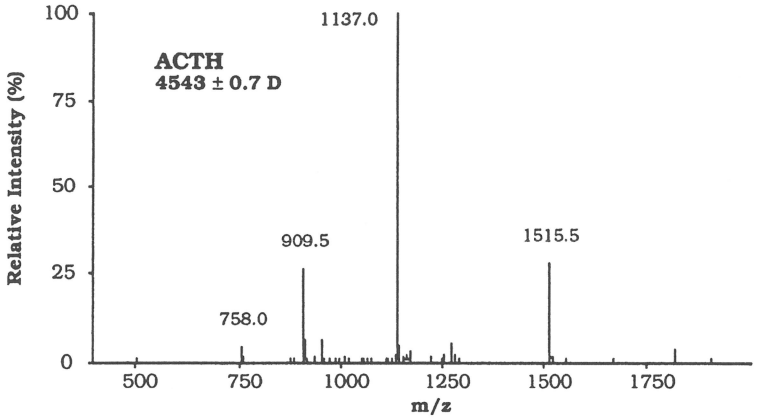
In: Donike, H. Geyer, A. Gotzmann, U. Mareck-Engelke, S. Rauth (eds.)
Recent Advances in Doping Analysis (1). Sport und Buch Strauß, Köln 1994

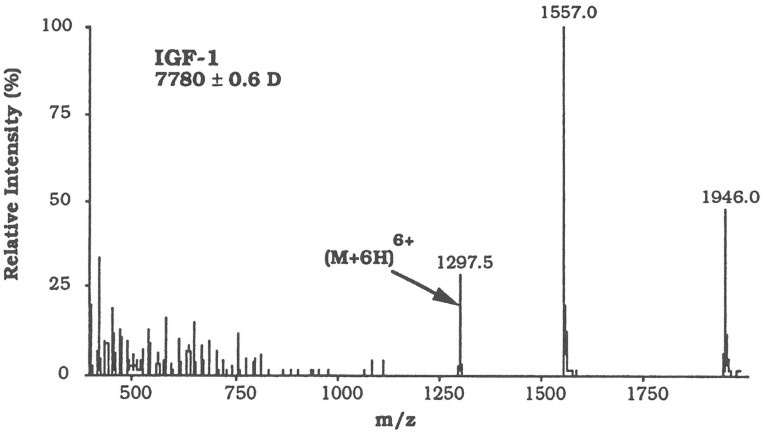
Myoglobin Spectra and Quantitation
(m.w. 16,695 D)





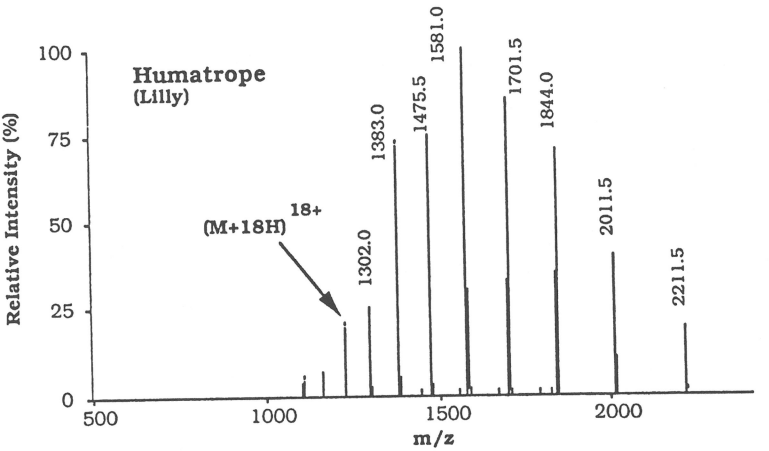
In: Donike, H. Geyer, A. Gotzmann, U. Mareck-Engelke, S. Rauth (eds.)
Recent Advances in Doping Analysis (1). Sport und Buch Strauß, Köln 1994

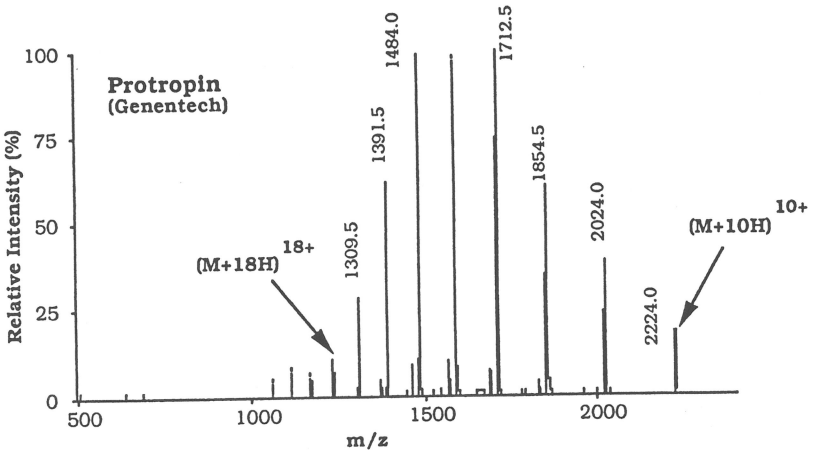




In: Donike, H. Geyer, A. Gotzmann, U. Mareck-Engelke, S. Rauth (eds.)
Recent Advances in Doping Analysis (1). Sport und Buch Strauß, Köln 1994

Comparison of hGH and Met hGH





In: Donike, H. Geyer, A. Gotzmann, U. Mareck-Engelke, S. Rauth (eds.)
Recent Advances in Doping Analysis (1). Sport und Buch Strauß, Köln 1994