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Analytical advances in detection of performance-enhancing compounds

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The use and abuse of performance-enhancing substances has been an issue in sports since the ancient Greeks. The availability of numerous synthetic steroids and recombinant peptide hormones has made testing an analytical challenge. Recent advances in mass spectrometry have provided an opportunity to decrease detection limits. The Atlanta Olympic Games in 1996 marked the first time every specimen was screened by gas chromatography (GC) coupled to high-resolution mass spectrometry (MS). A further improvement may be seen with GC/MS/MS and quadrupole ion traps. Electrospray HPLC/MS has also been applied to the detection and confirmation of peptide hormones in urine. The ability to detect subtle differences in oligosaccharide structure may provide a way to detect abuse of recombinant glycoproteins. Simply decreasing detection limits is not enough; new technology also allows development of a foundation on which to base interpretation. Application of HPLC/MS/MS has allowed direct measurement of steroid conjugates in urine. The relative importance of sulfate, glucuronide, and other conjugates and metabolites of testosterone and epitestosterone can now be assessed. In the international sports arena, the impact of genetic metabolic disposition must also be considered if we are to provide an equitable system. Further research will establish more-refined criteria for the detection threshold of abused substances.

INDEXING TERMS: sports medicine • abused drugs • drug assays • GC-MS • tandem MS • steroids • peptide hormones • urine • genetic variants

The use of performance-enhancing drugs in sport has been controversial since the time of the ancient Greeks. In the early 1960s, the Council of Europe banned the use of endogenous or exogenous agents to achieve an artificial and unfair increase in performance in competition. In general, the rationale for banning certain pharmaceutical agents is the desire for a "level playing field" and concern for the health of the athlete *[1]*. The attitude of some athletes, "The game... is to always be one up on the testers" *[2]*, makes continued improvement of testing imperative if a level playing field is to be assured. The International Olympic Committee (IOC)[1] has an extensive list of banned substances fitting into the general categories of stimulants, narcotic analgesics, anabolic agents, peptide hormones, and urine-manipulating agents.

The first testing for banned substances was carried out at the 1972 Olympic Games in Munich, where gas chromatography with nitrogen-selective detection was used to test >2000 urine specimens for stimulants. Although GC/MS was used to confirm the presence of anabolic steroids at the 1976 Montreal Olympic Games, a major advance in analytical methodology was implemented at the 1983 Pan American Games, where benchtop quadrupole GC/MS instruments were used to screen all urine specimens. Another potentially significant advance in analytical technology took place the 1996 Olympic Games in Atlanta, where GS/sector MS systems were used at moderate mass resolution to screen all of the urine specimens for anabolic agents.

With the ever-increasing number of dietary supplements used by athletes, as well as the pharmacological sophistication of their trainers and physicians, detection of performance-enhancing compounds banned by the IOC is increasingly difficult. The combination of "no-notice" testing of athletes (while out of competition) and at-competition testing with appropriate analytical approaches can minimize the opportunity to achieve benefit from banned substances. Here, I review some of the latest

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[1] Nonstandard abbreviations: CID, collision-induced dissociation; EPO, erythropoietin; ES, electrospray; GC/MS, gas chromatography/mass spectrometry; hGH, human growth hormone; hCG, human chorionic gonadotropin; HRMS, high-resolution mass spectrometry; IGF-1, insulin-like growth factor-1; IOC, International Olympic Committee; LOD, limits of detection; MS/MS, tandem mass spectrometry; QIT, quadrupole ion trap; SIM, selected-ion monitoring.

advances in analytical methods under development to test for drugs that affect athletic performance.

Materials and Methods

All materials were used as obtained without further purification. Sulfate and glucuronide conjugates of unlabeled (d_0) testosterone were purchased from Steraloids (Wilton, NH). Unlabeled (d_0) epitestosterone conjugates and all four 16,16,17-$^2H_3$-labeled (d_3) conjugates (used as internal standards) were synthesized in our laboratory. Ammonium acetate (purity 99.999%) and acetic acid (doubly distilled) were from Aldrich (Milwaukee, WI). Human growth hormone (hGH) preparations [Humatrope (Lilly, Indianapolis, IN) and Protopin (Genentech, South San Francisco, CA)] were obtained from the Indiana University Hospital Pharmacy. Insulin-like growth factor-1 (IGF-1) was obtained from Bachem Bioscience (King of Prussia, PA). High-purity (18 MΩ) water was obtained by passing house-distilled deionized water through a Barnstead Nanopure II system (Syborn/Barnstead, Boston, MA). HPLC-grade methanol was obtained from Curtin Matheson (Houston, TX).

All MS/MS work was carried out with a PE–Sciex (Perkin-Elmer–Sciex, Norwalk, CT) API III^{PLUS} triple-quadrupole MS equipped with Ionspray$^{™}$, a pneumatically assisted electrospray (ES) interface. To introduce the HPLC effluent, this laboratory uses a Beckman (Brea, CA) Model 126 programmable solvent module used in conjunction with a Rheodyne (Cotati, CA) 8125 injection valve housed in a DuPont (Wilmington, DE) forced-air oven. Quantitative data acquisition was done with the Routine Acquisition and Display program and the areas measured with the MACQUAN program (both PE-Sciex). Details of the experiments can be found in the relevant references.

Results and Discussion

ANABOLIC STEROIDS

Recent statistics from the IOC-accredited laboratories indicate that anabolic agents remain the primary performance-enhancing substances detected in athletes. A review of anabolic steroid metabolism has been published recently *[3]*. The analytical challenge in urine steroid analysis is threefold: the low concentrations of anabolic steroid metabolites (μg/L range); the large number of steroids and steroid metabolites sought; and the complexity of the urine matrix, which contains steroids of similar structure at concentrations as much as 1000-fold higher than the compounds of interest. A flow diagram of a commonly used approach for the analysis of anabolic agents is shown in Fig. 1; note that only the glucuronide conjugates of the steroids are routinely analyzed. The requirement for low limits of detection (LOD; 100 pg on-column) has mandated the use of selected-ion monitoring (SIM) GC/MS. A typical SIM method for anabolic agents incorporates 10–12 time-programed acquisition groups of 10–20 ions each. Sequential isolation of this

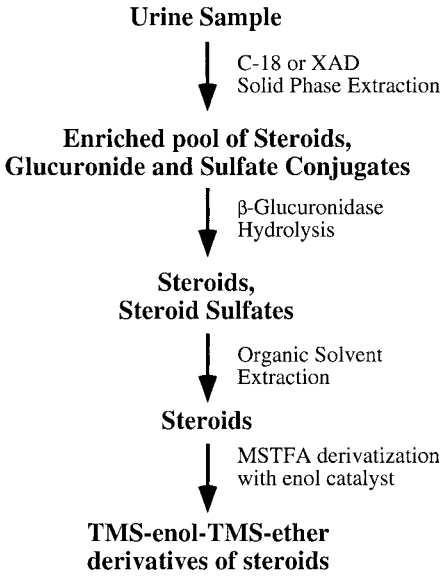

Fig. 1. Flow diagram of sample-preparation procedure for urinary anabolic steroid metabolites.

many ions, along with the incumbent instrument overhead time, is hindered by the sampling and signal-to-noise limitations of the mass filter.

In principle, the quadrupole ion trap (QIT) has an advantage over the quadrupole mass filter when large numbers of ions must be monitored. The QIT can be operated in three modes: selected ion detection; selected ion storage; and selected ion ejection. In selected ion storage, an ion of a single mass-to-charge ratio (m/z) is retained in the QIT at a time, much like SIM operation of a quadrupole mass filter. In the selected ion ejection mode, several ions of different m/z values can be stored simultaneously. The advantage of filling the QIT with only the ions of interest is improved limits of detection. Unfortunately, selected ion ejection techniques appear to be of limited benefit when the background is determined by the chemical composition of the biological matrix. In this case, the relative signal arising from the compound of interest can be increased, but the signal-to-noise ratio is not improved, given a similar increase in the background signal from compounds of similar mass *[4]*.

LOD can be improved through more-selective detection techniques, such as GC/high-resolution mass spectrometry (HRMS). Mass resolution (R) is defined as $M/\Delta M$, where M is the mass being detected and ΔM is the separation of the mass of interest from an adjacent mass. The ΔM can be measured at the half-height of the mass peak or at 10% of the peak height, depending on the inherent shape of the mass peak in the mass analyzer (e.g., quadrupole mass filter vs magnetic/electric sector). A quadrupole MS delivers unit mass resolution at half-height, with an R of ~500 at m/z 500. With an R of 5000, one can selectively detect ethanol (C_2H_4O, m/z 46.042) in the presence of formic acid (CH_2O_2, m/z 46.005) without chromatographic separation. The advantage of GC/

HRMS in the detection of anabolic agents is shown in Fig. 2, in which a urine sample supplemented with clenbuterol, 1 μg/L, was analyzed with the same GC/HRMS instrument but with adjustment of the mass resolution. The use of GC/HRMS for routine analysis of environmental dioxins has been required for a decade, and its advantages for isotope-dilution methods for cortisol analysis were recognized in the 1970s. The main disadvantages to GC/HRMS instruments are their relatively large size, the considerably increased costs for purchase and maintenance, and the degree of expertise required for their operation.

A similar enhancement in LOD might be expected from tandem MS (MS/MS), in which monitoring a selected product ion produced from the collision-induced dissociation (CID) of a precursor ion can provide exquisite selectivity *[5]*. Tandem MS can be performed either in space, with use of sequential sets of quadrupole rods, or in time, utilizing the storage capabilities of the ion trap. Using a Varian Associates (Palo Alto, CA) Saturn III QIT in the MS/MS mode and large-volume temperature-programed injection techniques, Borts and I have detected 100 fg of norandrosterone injected on-column, corresponding to a 6 ng/L concentration in urine *[6]*. The LOD achieved for other steroids will depend on the availability of a relatively intense, high-mass precursor ion *[6]*. Internal ionization ion traps (e.g., Varian Saturn III) and external ionization ion traps (e.g., Finnigan GCQ) appear to have some differences in LOD (J. Segura, personal communication). The application of GC/MS/MS technology to screening methodology will present some difficulties, because the sequential mass isolation and CID for large numbers of ions present the same sampling time limitations discussed above for SIM. It remains to be seen whether improved acquisition software can improve this situation.

Improved GC resolution could also improve the LOD, which is limited by the number of compounds extracted from the matrix. We postulated that the three-dimensional rigidity of the new generation of polysilarylene-bonded-phase capillary GC columns might improve selectivity between various steroid isomers; however, this hypothesis could not be confirmed *[6]*. Similar lack of improved selectivity was observed for polychlorinated biphenyls/dioxins in environmental testing (M. Hastings, personal communication), suggesting that, when the polycyclic backbone of the compound of interest is sufficiently large, the phenyl moiety in the stationary phase cannot be closely approached and thus cannot impart stereochemical selectivity. Application of improved chromatographic techniques, e.g., multidimensional GC *[7]*, could potentially be used with high-speed time-of-flight MS. Effective implementation of multidimensional GC requires the use of independent, orthogonal separation mechanisms *[8]*. Although the above-mentioned phenylmethyl-type columns do not provide orthogonality, the use of cyanopropyl- or trifluoropropyl-bonded phases based on the polysilarylene approach should provide different selectivities while providing the higher temperature limits and robustness required for routine steroid analysis.

Improved detection limits could also be achieved with improved sample clean-up strategies (see Fig. 1). Immunoaffinity extraction and HPLC preparation of steroids have been reported *[9]* but at present are difficult to apply to the screening of large numbers of samples. Detection of the residues of anabolic agent administration for long times after the administration requires not only sensitive

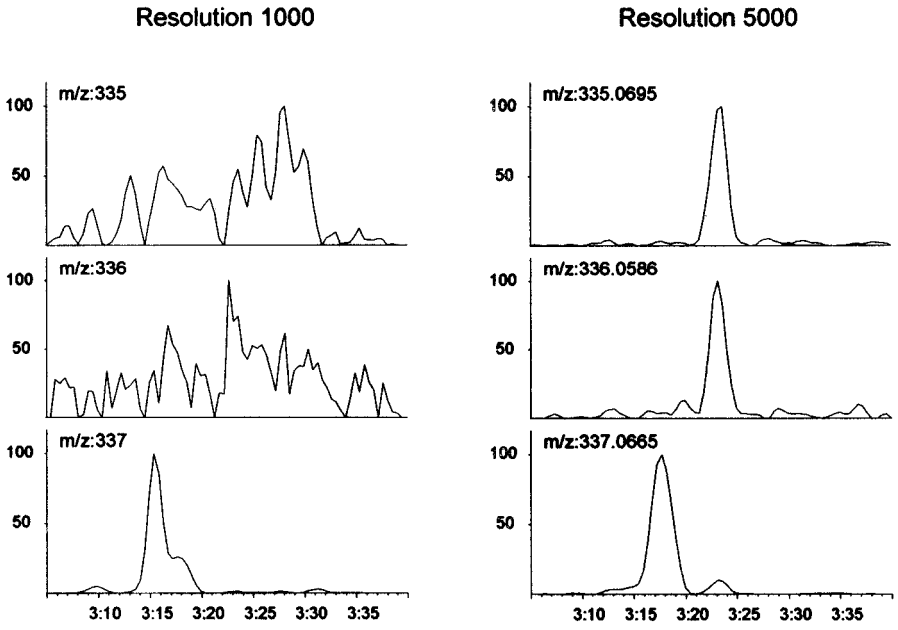

Fig. 2. GC/MS chromatographic trace for a urine sample with added clenbuterol, 1 μg/L, as determined with a Finnigan MAT 95S sector MS system, the mass resolution (R) being adjusted to either 1000 (*left panels*) or 5000 (*right panels*).

The *top* trace is the molecular ion (*m/z* 335; ^{35}Cl isotope), and the *center and bottom traces* are the M + 1 (*m/z* 336) and M + 2 (*m/z 337;* ^{37}Cl isotope) stable isotope, respectively. Note the large peak from an unrelated compound in the *bottom trace*. Source: Produced in collaboration with Stevan Horning, Institute of Biochemistry, Cologne Sports University, Cologne, Germany.

analytical techniques and appropriate timing of specimen collection but also selection of an appropriate long-lived metabolite *[3]*. For example, the chemical rearrangement of the sulfate metabolites of 17α-methyl-17β-hydroxy steroids to form 18-*nor*-17,17-dimethyl-13(14)-en compounds *[3, 10]* may provide a selective approach to the detection of this group of orally active anabolic steroids.

Although much progress has been made in detecting exogenous steroid use, detecting the use of normally occurring steroids (e.g., testosterone, dihydrotestosterone, and dehydroepiandrosterone) has been more difficult. A testosterone/epitestosterone (T/E) ratio >6 has been used to detect testosterone abuse *[11]*. Dehennin has suggested that many of the cases of what has been termed "naturally-elevated" T/E are attributable to the excretion of substantial amounts of epitestosterone sulfate *[12]*, a compound that would not be detected in the usual steroid procedure. We have developed a method for directly detecting the sulfate and glucuronide conjugates of steroid metabolites by using HPLC/MS/MS *[13]*. Deuterated internal standards are used for both conjugates of testosterone and epitestosterone (Fig. 3) because of the effect of the interface and collision energy on the resulting product ion production.[2] Four internal standards correct for any fluctuation in formation of the ammonium adduct used for sulfate conjugate detection and any fragmentation, as was observed for the glucuronide conjugates. The results to date suggest that about one-third of the cases of "naturally elevated" T/E are the result of unusually high concentrations of epitestosterone sulfate and low concentrations of epitestosterone glucuronide. In the course of our studies, we verified that concentrations of testosterone glucuronide in the urine of Chinese men are low, although the amounts of testosterone sulfate are similar to those of Caucasian men.[2] The use of direct detection of the steroid conjugates with HPLC/MS/MS provides a powerful tool to investigate stage II metabolism of steroids, and studies are underway in our laboratory to (*a*) understand the racial differences in testosterone metabolism and excretion and (*b*) identify additional metabolites that indicate exogenous use.

Another promising approach to detecting the use of exogenous testosterone has been the use of GC/combustion/isotope ratio MS *[14, 15]*. The premise of this approach is that the ^{13}C abundance of a pharmaceutical preparation of testosterone is differs from that in testosterone produced from dietary precursors. A comparison of the ^{13}C abundance of testosterone with that of its metabolic precursors that indicated a difference in abundance would support a finding of a nonmetabolic source of testosterone. Although preliminary results appear promising, much work must be done to validate this new methodology. In particular, the detection of exogenous use in the presence of a mixture of trace amounts of native and exogenous testosterone will be a challenge.

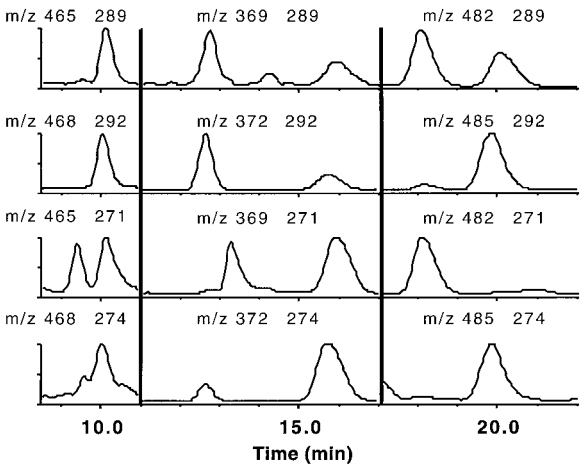

Fig. 3. HPLC/MS/MS selected reaction monitoring profile for testosterone and epitestosterone glucuronides and sulfates.

The precursor ion mass is shown to the *left* of the *arrow*, and the product ion mass is shown to the *right* of the *arrow* for each time-programed acquisition segment. The chromatographic traces show the ions collected for CID product ions of both native (465, 369, and 482 parent ion *m/z*) and deuterated (468, 372, and 485 parent ion *m/z*) steroid conjugates. The elution order of the compounds is testosterone glucuronide, testosterone sulfate, epitestosterone sulfate, and epitestosterone glucuronide (Bowers, unpublished data).

In our increasingly litigious society, athletes rely on such defense strategies as the effect of ethanol on the testosterone/epitestosterone ratio *[16]*, inadvertent ingestion *[17]*, and bacterial contamination to attempt to avoid the penalties for detection of banned substances in their urine. In most circumstances, the laboratory has methods at its disposal to refute these challenges. Nevertheless, improved LOD alone will not resolve these issues, and research is underway in several laboratories to provide the appropriate pharmacological foundation for further testing.

PEPTIDE HORMONES

The IOC banned substance list includes adrenocorticotropin, hGH, chorionic gonadotropin (hCG), erythropoietin (EPO), and their releasing factors. Although detection of these peptides by immunoassay is well established in the clinical laboratory, the acceptability of this method of analysis as definitive proof of administration has yet to be established in forensic toxicology. Recent advances in the generation of ions directly from liquids has suggested the possibility of confirmation of peptide hormones by MS. The use of ES ionization allows the production of multiply charged ions from peptides and proteins, which extends the effective mass range of the mass spectrometer. Fig. 4 shows the ES mass spectrum of hGH from Genentech and Lilly. The addition of a single methionine to the carboxy-terminal end of the Genentech peptide allows a clear differentiation of the mass of the two proteins, distinguishing between 22 124 Da and 22 255 Da. Thus, detec-

[2] Sanaullah, Borts DJ, Bowers LD. Direct measurement of testosterone and epitestosterone conjugates in urine by HPLC/MS/MS. Manuscript in preparation.

tion of the recombinant hGH in the presence of native protein would be relatively easy. The second issue for MS confirmation of peptides in urine is the LOD. Little trace quantitative work of proteins has been done with HPLC/MS. We applied three-ion SIM analysis to the HPLC/MS detection of IGF-1. An HPLC/MS trace for injection of 10 fmol of IGF-1 is shown in Fig. 5 *[18]*, establishing that detection limits with ES HPLC/MS should not be a problem.

Determination of glycoproteins such as hCG and EPO presents additional challenges because of the presence of oligosaccharide moieties. The microheterogeneity of the oligosaccharides gives rise to a large number of molecules that all have the same amino acid sequence but different masses and charges. This precludes direct monitoring of the intact peptide by use of ES ionization. Monitoring the unique tryptic digest fragments provides an opportunity to identify peptide hormones. Both ES ionization HPLC/MS *[19]* and matrix-assisted laser desorption ionization–time-of-flight mass analysis ("MALDI-TOF") *[20]* have been applied to detection of hCG. Whether either or both techniques have the ability to produce quantitative as well as qualitative identification data remains to be established. Enrichment of peptide hormones from urine by immunoaffinity trapping can be readily achieved *[21]*, which should facilitate trace determination of the peptide hormones. Differences in the electrophoretic mobility of native and recombinant EPO were used to detect administration of the latter in 11 of 15 urine samples *[22]*. Work is currently underway in our laboratory to determine whether the observed charge differences can be exploited to develop an HPLC/MS method for detection of recombinant EPO.

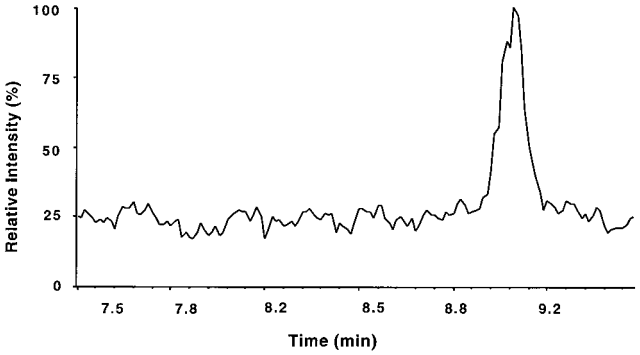

Fig. 5. SIM HPLC/MS chromatogram of 10 fmol of IGF-1 *[18]*.

Numerous issues surround which markers are necessary for identification of peptides. For example, the ions used for SIM analysis of IGF-1 mentioned above are multiply charged species formed by multimolecular reaction dynamics at atmospheric pressure. This is distinctly different from the electron ionization unimolecular decomposition reactions that are widely accepted as indicative of structural features in GC/MS analysis. The reproducibility of ion ratios for ES ionization must be established. In addition, the number of ions necessary to establish the "identity" of the peptide has not been tested, and the use of tryptic digests raises the issue of how many tryptic fragments are necessary to establish identity of a protein. Clearly, the combination of immunoaffinity extraction from urine with detection by MS increases selectivity, but the extent to which it should relax the above criteria must be determined. Although these analytical standards must be established in the laboratory, the legal admissibility of MS confirmation of peptides and proteins will be established by precedent.

In summary, application of state-of-the-art analytical tools to detection of performance-enhancing compounds will decrease the limits of detection and increase the numbers and types of compounds that can be detected. Analytical advances will not only benefit athletic drug testing, but should provide new methods for, e.g., characterizing protein standard materials *[19]*. Pharmacokinetic and pharmacodynamic data must also be accumulated to support the analytical advances, so that fair and equitable medical review of cases can be undertaken. Given the amount of financial resources invested in sport, the athletes (and fans) have the right to competition decided by innate ability and hard work—not by potentially dangerous pharmacological intervention.

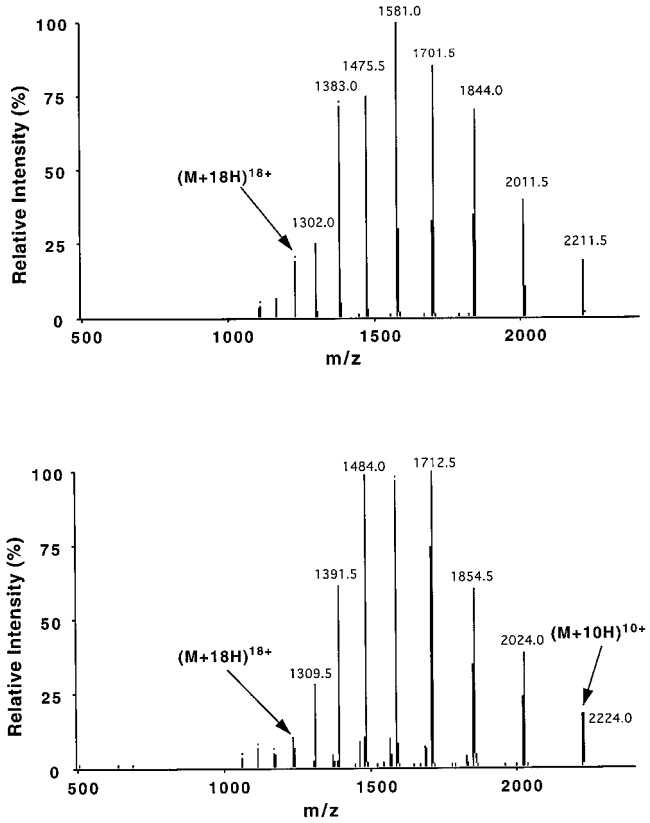

Fig. 4. ES mass spectrum of Genentech (*top*) and Lilly (*bottom*) recombinant human growth hormone *[18]*.

Ions shown are the multiply charged species produced, 10 to 18 charges per molecule.

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