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The Potential Molecular Marker In Distinguishing Exogenous Recombinant Human Growth Hormone Using MALDI-TOF-MS/MS

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Introduction

Growth hormone was banned by IOC and WADA [1] in the 1990s. However, due to its pulsive secretion rhythm, GH concentration might be influenced by gender differences, age and stress circumstances. Thus it was difficult to establish a normal range among healthy population and athletes. In addition, the very short half time of the rh-GH and extreme low abundance in the body fluids (ng/mL in serum, pg/mL in urine) as well as a series of heterogenous molecular isoforms hindered the development of both indirect and direct detection methods. There have been several papers using ratios of GH variants to distinguish exogenous GH[2, 3]. However, these methods were based on immunoassays, and the antibodies might exist cross-reaction between GH and other species protein. Therefore, it was necessary to combine mass spectrometry to detect GH. In the context, the goal of the present study was to explore the molecular markers to distinguish endogenous and exogenous GH using MALDI-TOF-MS methods. Four rh-GH preparations made in China were analyzed using MALDI-TOF-MS/MS.

Experimental

Sample preparation and MALDI-TOF-MS/MS analysis

Four types of rh-GH preparations were purchased from Chinese pharmaceutical manufacturers (Ansomone®, Genheal®, Gensci®, Sigrow®) and were randomly coded with number 001 to 004. Sensitivity experiment was conducted using a series of rh-GH solutions prepared, which was diluted by matrix solution. The minimum load amount was 1.625ng. Matrix solution used α-cyano-4-hydroxy cinnamic acid (CHCA, 5 mg/mL, Sigma) diluted in

acetonitrile (AcCN, 50%, v/v, Sigma) solution containing trifluroacetic acid (TFA, 0.1%, v/v). The rh-GH solutions were diluted by matrix solution (1:10, v/v), and a mixture of 0.5 µL was dropped on the stainless steel plate for MALDI-TOF-MS analysis.

Tryptic digestion

The rh-GH solution was diluted to 1mg/mL. GH solutions of 20 µL was added in a 0.5 mL Eppendorf vial and freezed to dryness under vacuum. Sample was added 10 µL buffer (1M Tris-HCl pH 8.0, 8M Urea, 1M DTT) at 37°C for 4 hours then added 25mM iodoaceticamide and kept for 1 hour avoid light. Trypsin (Promega, concentration of 1ug/uL, 1:50, w/w) and ammonium bicarbonate solution (90µL 20 mM) was added. Digestion time was 12 hours.

MALDI-TOF-MS/MS

Mass spectra in positive ion mode were acquired on 4700 Proteomics Analyzer, (Applied Biosystems, USA). Molecular weight was determined using Linear High Mass method. Mass calibration was conducted using the Calibration Mixture 3 (Lot No. 0308012, Foster City, CA 94404, USA). MS/MS were resolved using the GPS software and the Mascot database.

Results and discussion

The mass spectra of rh-GH using MALDI-TOF-MS are shown in Figures 1 and 2.

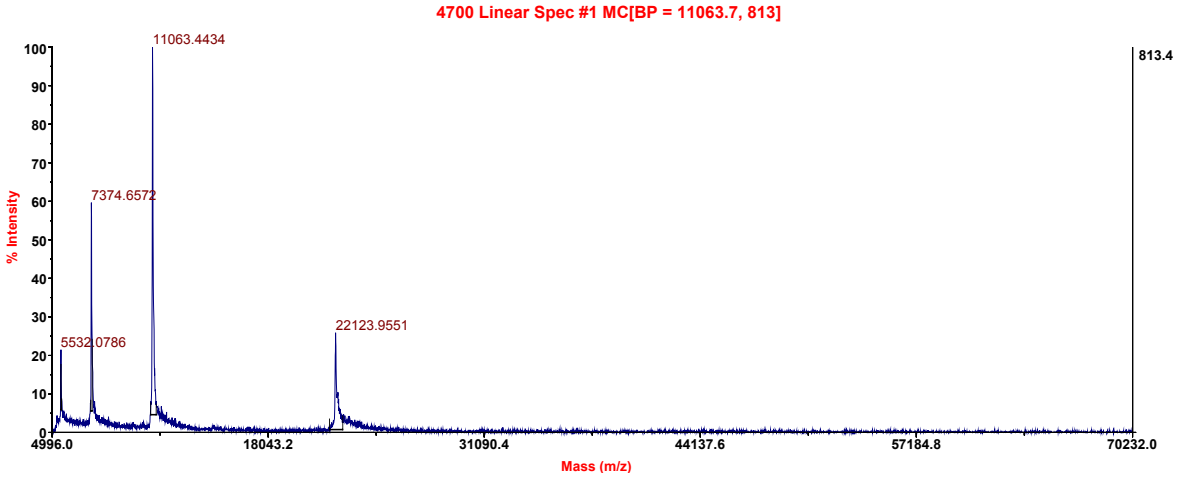

Figure 1. The MALDI-TOF MS of rh-GH, absolute load amount was 1.625ng rh-GH, about 74fmol. The molecular weight was 22124 Daltons (Manufacturer's Code was 002), the theoretical MW was 22124.1 Daltons, (CAS-12629-01-5).

The preparations' MW were 22124 (Figure 1), 22255 (Figure 2), 22114 and 22116 (not shown) Daltons, respectively. The mass differences with theoretical value was 0.1, 130, 10, 8 Daltons respectively. One of the rh-GH preparations showed that the MW was 22255 Daltons, in Figure 2, the mass difference with the theoretical value 22124.1 was 131 Daltons, which closely corresponded with the methionine residue 131 (monoisotopic mass). Therefore, it

indicated that the preparation (Manufacturer's Code was 001) contained an extra methionine residue than the other three preparations. To confirm the findings, peptide products after trypsin digestion were analyzed using MALDI-TOF/MS/MS (Figure 3). The mass spectra result was consistent with the fact that there existed a methionine residue on the N-terminal of the first tryptic digestion peptide of GH (T1).

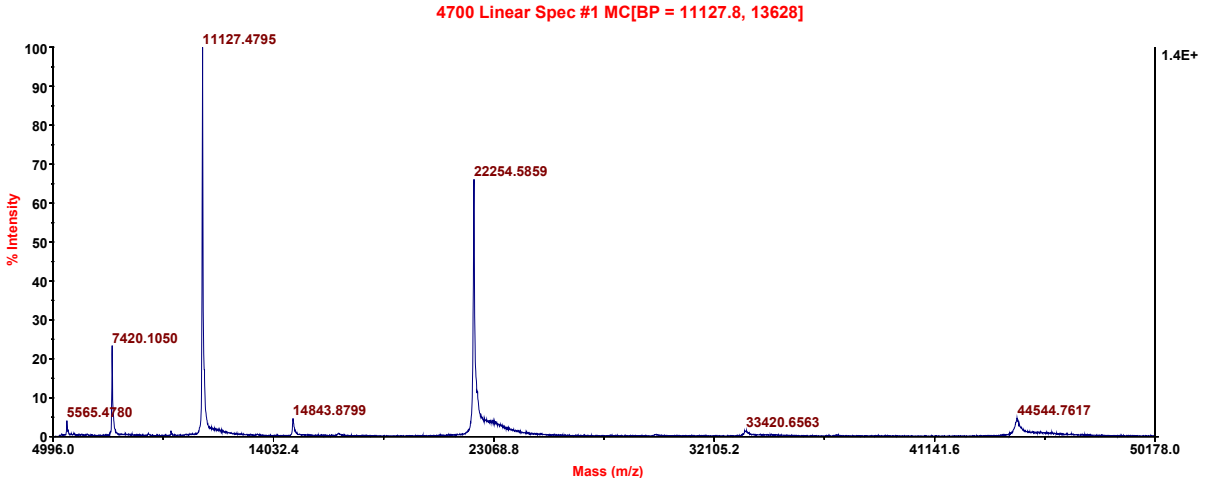

Figure 2. One type of rh-GH preparation's MS (Manufacturer's Code was 001)

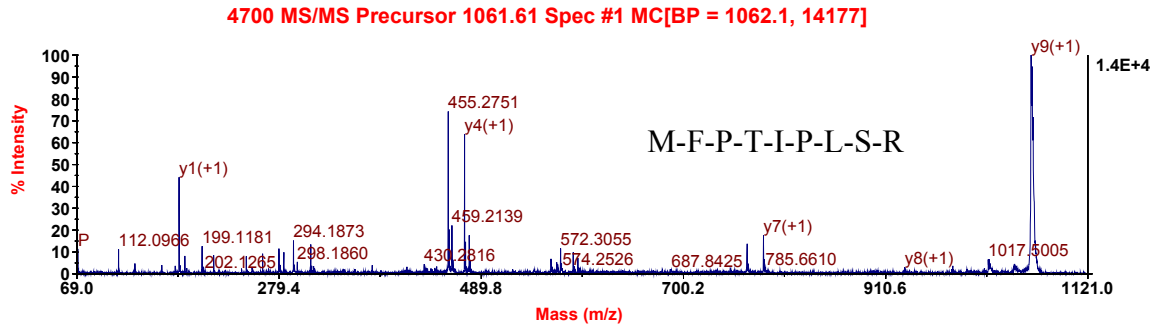

Figure 3. MS/MS fragmentation of the peptide labeled with the major y ions, tryptic digestion of the methionic rh-GH's (MW=22254) first peptide.

According to the N-terminal's methionine status, four rh-GH preparations can be classified as two groups, one group had the correct peptide sequence identical to the endogenous 191 amino acids, the other had the extra methionine on N-terminal, which had 192 amino acids. The reasons why was because gene expression mode would affect the location of the recombinant preparation. When expressed in E. coli, promoters and terminators was required to initiate translation procedure. Usually An ATG or GTG preceded the target gene sequence [4]. Thus the primary preparations owned a methionine residue on its N-terminal. For eukaryotic cell line, there existed the post-translational modification mechanism to remove signal peptide and got the non methionyl recombinant preparation. Therefore, the methionine on the N-terminal of rh-GH can be used as the molecular marker in distinguishing with the endogenous GH. The 22KD methionyl rh-GH was produced in 1981 [5] and 20KD methionyl

rh-GH in 1985 [6]. This methionyl rh-GH was evaluated biological effects in the clinical trails in 1986. However, some pharmaceutical manufacturers had adopted certain techniques such as using cyan bromide, methionine aminopeptidase [7] even changing expression host, to produce the rh-GH which had the identical primary structure with the endogenous growth hormone.

It was noted that there still existed some methionyl recombinant GH preparations on the clinical markets, which had the possibility to be abused by athletes. Therefore, it should be highlighted to the doping control laboratories in China and abroad. We proposed the ideas of new procedure in testing rh-GH in our laboratory. Firstly, using immunological tests to screen the blood samples. Secondly, once the GH concentration was higher than the cutoffs, samples should be further analyzed using the separation and enrichment techniques including immunoaffinity chromatography and electrophoresis techniques. Thirdly, MALDI-TOF-MS/MS was used to confirm the results. We hope the method should be further evaluated by other doping control laboratories and aroused attention in the practice of antidoping.

Conclusion

(1) The sensitivity of MALDI-TOF-MS could detect 1.6 ng（73 fmol） using the intact molecule of rh-GH. (2) Four preparations of rh-GH from different pharmaceutical corporations were analyzed using MALDI-TOF-MS/MS. One rh-GH's molecule had the extra methionine on its N–terminal, which could be used as the molecular marker to distinguish with the endogenous GH. (3) When 50 ng (2 pmol) rh-GH was analyzed, the peptide recovery rate was 36% retrieved by Mascot database.

Acknowledgement

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