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Growth Hormone in Biological Fluids: What about using Surface Plasmon Resonance

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ABSTRACT

The abuse of recombinant human growth hormone in various sport disciplines has been suspected for many years but no solid analytical method, capable of corroborating these suspicions has been developed. The most promising entries to such method include the use of immunoglobulins with distinct specificities for particular growth hormone isoforms. As such, differential ELISA-based approaches are being developed in which the ratio between two or more growth hormone isoforms are evaluated. These approaches require at least two antibodies of distinct specificities, immobilised to the solid support and, at least, one additional, labelled, broad-specificity antibody to permit detection and quantification. Here we present a different approach to the same purpose, based on the optical phenomenon of surface plasmon resonance. This technique allows evaluation in real time of biomolecular interactions taking place under dynamic conditions and without the stringent requirement of a labelled secondary antibody. In addition the approach includes the recovery of interacted material for posterior mass spectrometric characterisation. An introduction to the phenomenon of surface plasmon resonance is given as well as the preliminary results obtained.

INTRODUCTION

Recombinant 22 kDa human growth hormone (hGH) therapy, replacing human pituitary derived GH therapy, for the treatment of established growth hormone deficiency in the young was introduced in 1985 by Genentech (Protropin®) Since then all major pharmaceutical companies have included this recombinant substance in their product-list. A quick survey in world wide web suggests that this protein is easily available and the abuse of growth hormone (GH) as a possible performance-enhancing agent has therefore been suggested to be a common practice in sport. Suggested, as no truly reliable analytical tool currently exists for this purpose. Human, pituitary derived, growth hormone consists of a mixture of different "isoforms". Several molecular weight

variants[1] have been described including 22 kDa (~80-90%), 20 kDa (~5-10%), 17 kDa, 12 kDa, and 5 kDa that originate from full-gene transcription, alternative gene-splicing, and proteolytic cleavage. The heterogeneity of the growth hormone family is further enlarged through the occurrence of post-translational modifications such as glycosylation[2], *N*-acetylation[3], phosphorylation[4], and deamidation[5]. Finally, the complexity of the system is amplified by the formation of GH-oligomers and GH-GH binding protein complexes in circulation.

Several strategies have been pursued based on either direct or indirect evidencing of possible GH doping. Direct approaches as proposed up to now are based on the detection of GH concentrations, and rely on accurate measurement of the ratio between either 22 kDa GH *vs* 20 kDa GH[6] or 22 kDa GH *vs* non-22 kDa GH[7] through quantification of the interaction of different GH-isoforms with specific antibodies in ELISA-type settings. However, questions arise on whether the interaction of several different antibodies can be compared in a straightforward manner, how other GH-isoforms contribute to such ELISA read-out, and what the contribution of non-specific binding is.

The technique of surface plasmon resonance (SPR) may provide the answer to these questions and as such present a valuable alternative to ELISA based approaches. Surface plasmon resonance (SPR) is an optical technique used to monitor biomolecular interactions in real time and under dynamic conditions. One biomolecule (*e.g.* an antibody) is immobilised onto the sensor surface. Its binding partner (an antigen) is injected over this biomolecule in an aqueous buffer under carefully controlled conditions such as: continuous flow, buffer composition, and temperature. Detection of a biomolecular interaction is based on the optical phenomenon of total internal reflection (TIR) of a monochromatic and plane-polarised beam of light (Figure 1).

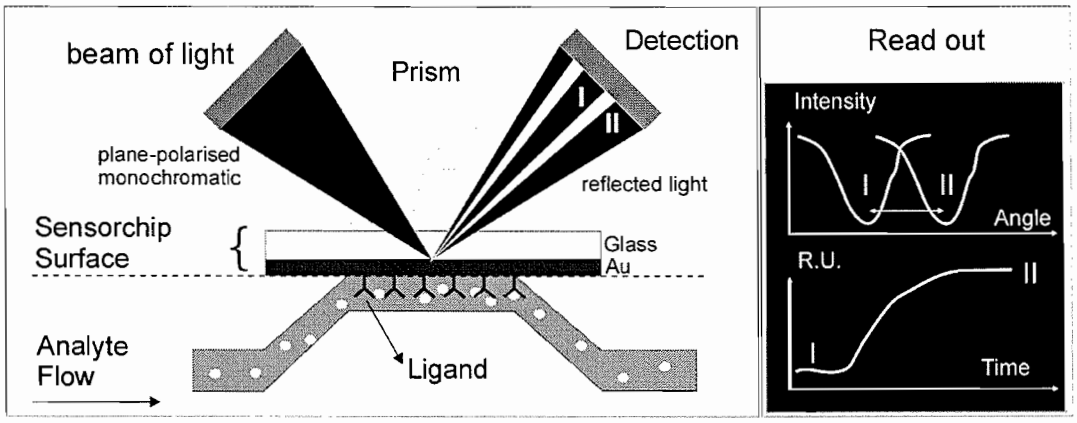

Figure 1. Explanatory scheme of the principles of SPR. A plane-polarised, monochromatic beam of light is reflected internally (TIR). At the opposite site of the inceding beam biomolecules are immobilised to a gold-surface (situation **I**). Upon interaction with a second biomolecule the SPR-angle changes (situation **II**) which is transformed into a sensorgram.

At a particular angle (SPR-angle) the intensity of the reflected light is decreased as photons interact with the gold-layer electrons to generate plasmons and an evanescent wave that penetrates into the opposite direction of the incident beam of light. The SPR-angle depends on the material at the surface of the gold layer. If an interaction takes places, the material at the surface changes and so does the SPR angle. These changes, representing biomolecular interactions, are registered *vs* time in a so-called sensorgram. The read-out is in Resonance Units (RU), where the equivalence of 0.1° angle change is about 1.000 RU or 1 ng/mm^2 of biomolecule.

A typical sensorgram has two distinct phases (Figure 2.); an association phase when antigen is injected and the interaction takes place, and a dissociation phase when injection stops and the read-out corresponds to a semi-permanent change at the surface *i.e.* the antigen attached to the antibody. Dissociation of the antigen from the antibody is accelerated through the application of a regeneration phase. Acidic, basic or saline solutions can be applied to the surface to separate the antigen from the antibody and recover the original state of the surface for re-use. Both quantitative data (concentration) as well as qualitative data (kinetic paramenters) can be derived from a sensorgram.

The instrument used in the present study (Biacore) allows the simultaneous monitoring of the interaction of four different antibodies with injected analyte through four different flow-cells. As such, using one flow-cell as control surface, non-specific, matrix-related interactions can be filtered out.

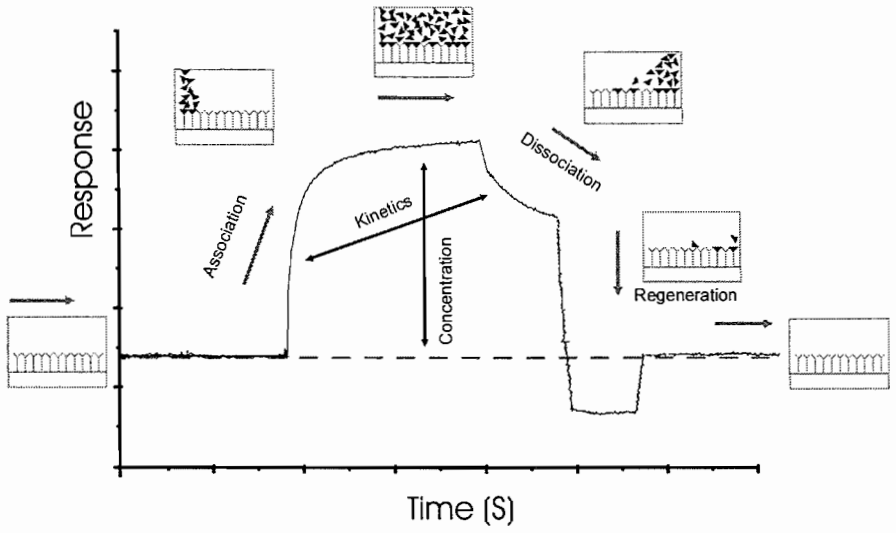

Figure 2. Typical sensorgram where analyte associates to the antibody while flow contains the antigen (association phase), where analyte dissociates the antibody (when flow is only buffer) approaching a stationary state (dissociation phase), and a regeneration phase to recover original state of antibodies (acid or basic conditions, with or without ionic strength or with complexants like EDTA).

Thus, this approach appears ideal to monitor, both qualitatively and quantitatively, the presence of different growth hormone isoforms in biological fluids. Finally, another important characteristic of the instrument used focuses on the possibility to recover samples (antigens trapped by the antibodies) for analysis by mass spectrometry (*e.g.* MALDI), obtaining an unequivocal determination of the antigen.

Here we describe the use of SPR as a differential immuno-sensing system applied to the problem of growth hormone analysis, the characterisation of different antibodies, and the mass spectrometric analysis of different recombinant and pituitary derived growth hormone isoforms.

EXPERIMENTAL

Materials - Monoclonal anti-GH antibody (clone n^0 A36020047P) was purchased from Biospacific Corporate (Emeryville, CA, USA). Monoclonal anti-20 kDa antibody (D05) was provided by Mitsubishi Kagaku Bioclinical Laboratories (Tokyo, Japan). Monoclonal goat-anti-mouse antibody was purchased from Pierce (Rockford, IL, USA). Monoclonal anti-β2-microglobulin was from Biacore (Uppsala, Sweden). Recombinant growth hormone from E. coli was obtained from Pharmacia & Upjohn (Barcelona, Spain; 22 kDa) and from Mitsubishi Kagaku Bioclinical Laboratories (20 kDa). Recombinant growth hormone (22kDa) from CHO cells was obtained from Serono Laboratories (Barcelona, Spain;). All recombinant preparation were pharmaceutical grade. Pituitary derived growth hormone standard material (80/505) was purchased from the National Institute of Biological Standards and Controls (NIBSC, United Kingdom).

Surface Plasmon Resonance analyses were performed on a BIACore 3000 instrument (Uppsala, Sweden). Sensor chips used in experiments were high-density carboxy-methylated dextran (CM5) sensor chips. Sensor chips containing specific antibodies were stored in a desiccator at 4 °C. Standard running buffers were based on HBS-EP (HEPES 10 mM pH 7.4, modified when necessary with NaCl 150 mM, EDTA 1 mM, and 0.005% (v/v) Polysorbate 20 (Tween 20)). All these components were purchased from BIACore (Uppsala, Sweden). SPR analyses were done at 25 °C at the typical flow rate of 5 µl/min, and employing a sample volume of 15 µl unless stated otherwise. All other reagents used were of the highest quality commercially available.

Immobilisation of antibodies or hGH isoforms on the surface was performed basically as described by Howell and Johnsson[8,9]. Coupling of the biomolecule of interest was achieved through the primary amine groups employing solutions of 50-60 µg/ml ($2.3\text{-}3\times10^{-6}$ M) for high level immobilisations and 5-10 µg/ml ($2.3\text{-}5\times10^{-7}$ M) for low level immobilisation in 10mM sodium acetate buffer (coupling buffer, pH 5.5).

Methods – Typical SPR analyses were performed employing pure solutions of single isoforms or mixtures of different isoforms. A series of different concentrations were evaluated allowing the determination of the linear range of the antibody as well as the detection limit. Kinetic experiments were performed on low-density antibody surfaces and five different concentrations (centred around the K_D of the antibody) of the appropriate isoforms. All sensorgrams were processed with the biaevaluation software (Biacore, Uppsala, Sweden)

One dimensional SDS-polyacrilamide gel electrophoresis was performed according to the method described by Laemmli[10]. Proteolytic digestions in solution were performed by incubating 1 µg of protein in 50 µl ammonium bicarbonate (100 mM, pH 7.8) with 0.02 µg trypsin for 16 h at 37 °C in a heater block. For in-gel digestions excised bands were washed with 150 µl demineralised water for 5 min, dehydrated with acetonitrile for 10 min and dried in a Speedvac. Reduction of the disulfide bonds was carried out by swelling the gel pieces in dithio thrietiol (10 mM in 100 mM NH_4HCO_3, pH 7.8, for 30 min at 56 °C. Prior to alkylation with iodoacetamide (55 mM in 100 mM NH_4HCO_3, 30 min in the dark) gel pieces were dehydrated once more. Next, gel pieces were washed thoroughly with 100 mM NH_4HCO_3 (4 x 150 µl), dehydrated with CH_3CN, and finally dried in a Speedvac. Rehydration was done with 100 mM NH_4HCO_3 containing trypsin (4 pg protease per ng protein), at 4 °C for 30-45 min. The enzyme solution was replaced by 25 µl 100 mM NH_4HCO_3 and incubated overnight at 37 °C.

Positive ion mode MALDI TOF mass spectrometric analyses of the native proteins and tryptic digests were performed on a Voyager-DE™ STR Biospectrometry workstation (Applied Biosystems, Foster City, CA, USA), equipped with a N_2 laser (337 nm). Typically, spectra were acquired using an accelerating voltage of 23.5 kV (grid voltage 90%, ion-guide wire 0.15%), a pulse delay of 150 ns for peptides and 600 ns for proteins, and accumulating 100 mass scans in the range 1-5, or 3.5-70 kDa, respectively. External calibration of the spectrometer was performed using bovine albumin for proteins and a mixture of Calmix I and II (Applied Biosystems, Foster City, CA, USA) for peptides. All recorded data were processed using Data Explorer™ Software (Applied Biosystems, Foster City, CA, USA).

RESULTS AND DISCUSSION

The results presented in the following section merely serve to demonstrate the potential of SPR in this field and by no means represent a complete study.

For the characterisation of the first antibody, A36020047P, a high-density surface was employed and different GH isoforms allowed to interact. This antibody displayed remarkable specificity for the 22 kDa isoforms (Figure 3.)

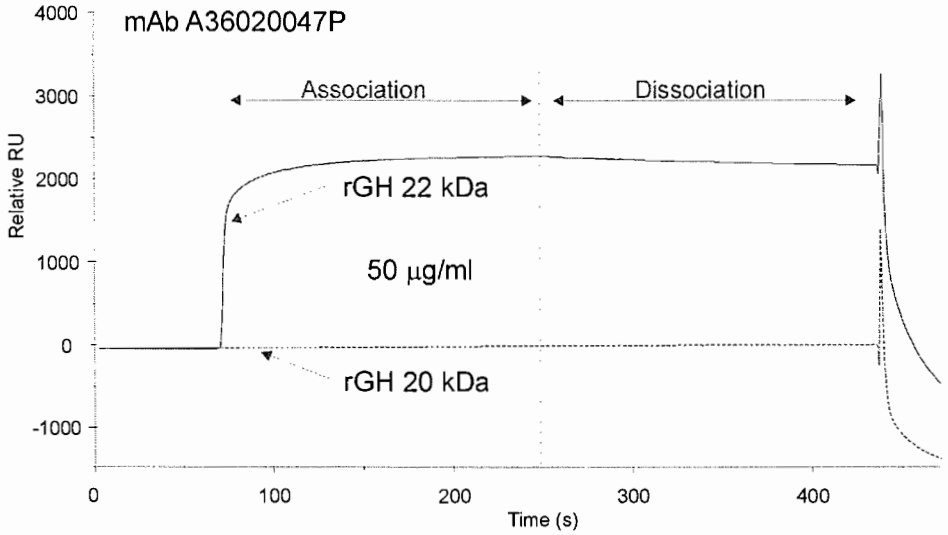

Figure 3. Sensorgram of 20 kDa (2.5 µM) and 22 kDa GH (2.3 µM) isoforms with mAb A36020047P. The specificity of this antibody for the 22 kDa isform is clearly evidenced.

The absolute absence of cross-reactivity of this antibody for the 20 kDa isoform is clearly evidenced in time and renders this antibody a valuable candidate for the specific detection of the 22 kDa isoform. For the evaluation of the linear range and limit of detection a series of 15 different concentrations were passed over the antibody giving rise to the sensorgrams as depicted in figure 4A and 4B.

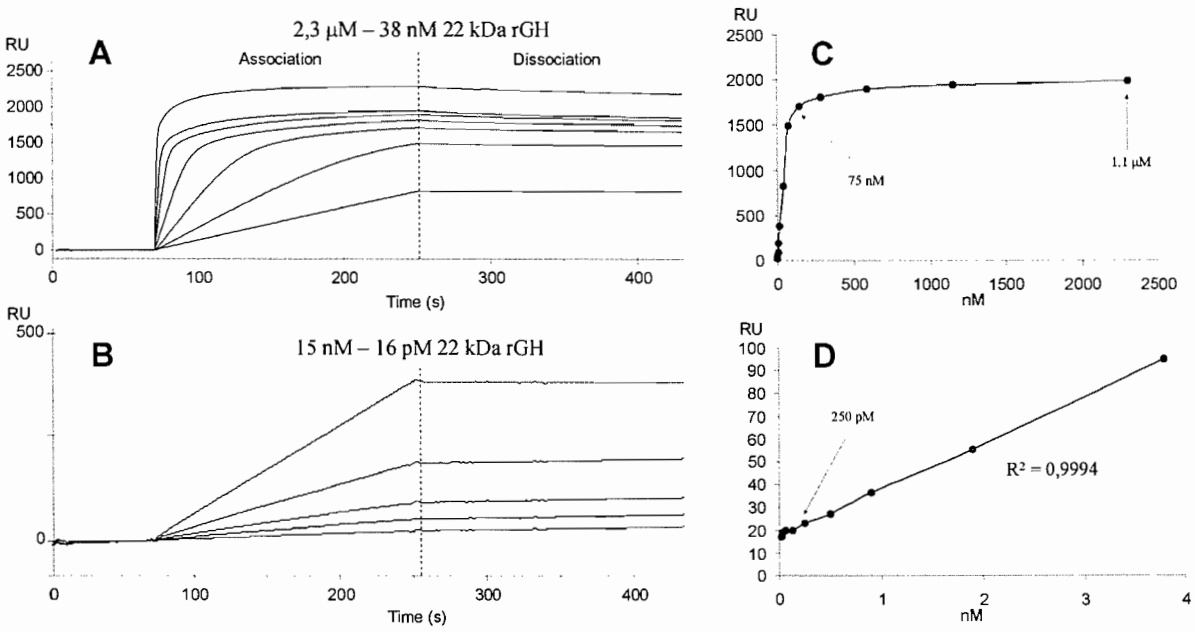

Figure 4. Sensorgrams (A and B) corresponding different concentrations of 22 kDa GH, interacting with A36020047P immobilised at the surface. Panels C and D (amplification of D) represent the concentration vs response curves for both series (A and B, respectively). The response represented in the curves corresponds to the response five seconds into the dissociation phase.

From the concentration *vs* response curves the linear range for this antibody was determined to be from ~1 to 75 nM. The limit of detection (LOD) was set at the concentration of 0.25 nM (5.5 ng/ml). With these data, and relying on literature data of average GH concentrations in urine, the detection of 22 kDa GH in this biological fluid would require a sample handling similar to that employed for the detection of erythropoietin. In the case of plasma, where reported values are a factor of 500-1000 higher, sample work-up could be less laborious. Finally, kinetic parameters of the interaction mAb – 22 kDa GH were determined employing a low-density sensorchip surface, five different 22 kDa GH concentrations and 5 different flow-rates (data not shown). The parameters obtained are summarised in table I.

Table I. Kinetic parameters obtained for the monoclonal antibody A36020047P employing 22 kDa GH at different concentrations and a flow-rate of 50 µl/min. Global refers to the analysis of the curves corresponding to all concentrations. Local refers to the analysis of a single concentration.

Analysis	[22kDa]	k_a(1/M.s)	K_d(1/s)	K_A (1/M)	K_D (M)	Rmax (RU)	$\chi 2$
Global	---	**$3{,}70\times10^{6}$**	**$3{,}92\times10^{-4}$**	**$9{,}44\times10^{9}$**	**$1{,}06\times10^{-10}$**	---	**0,0667**
Local	6,68nM	$3{,}41\times10^{6}$	$5{,}48\times10^{-4}$	$8{,}70\times10^{9}$	$1{,}15\times10^{-10}$	35,3	0,05395
Local	5,36nM	$3{,}90\times10^{6}$	$4{,}76\times10^{-4}$	$9{,}95\times10^{9}$	$1{,}01\times10^{-10}$	34,2	0,05395
Local	4,00nM	$3{,}88\times10^{6}$	$2{,}96\times10^{-4}$	$9{,}90\times10^{9}$	$1{,}01\times10^{-10}$	36,0	0,05395
Local	2,68nM	$4{,}62\times10^{6}$	$1{,}74\times10^{-4}$	$1{,}18\times10^{10}$	$8{,}49\times10^{-11}$	35,2	0,05395
Local	1,32nM	$5{,}35\times10^{6}$	$3{,}00\times10^{-4}$	$1{,}37\times10^{10}$	$7{,}32\times10^{-11}$	34,9	0,05395

These parameters, especially the association-rate constant, dissociation-rate constant and dissociation constant provide excellent tools to determine which other antibody, with a distinct specificity, should be used in conjunction when determining the ratio of GH isoforms present in a particular sample. If the antibodies were to have large disparities in these values, the response obtained at a particular time-point for at least one antibody would not truly reflect the concentration present and therefore result in erroneous readings.

A second antibody studied, DO5, clearly showed the importance of a proper characterisation of these molecules prior to their employment in any type of analysis. When the response of this antibody, immobilised at the surface at a similar density as A36020047P, to a high concentration of 20 kDa GH was contrasted with the response of the 22 kDa GH with A36020047P under comparable conditions, the former resulted in a relative response of only 6% (~115 RU *vs* ~2000 RU; Figure 5).

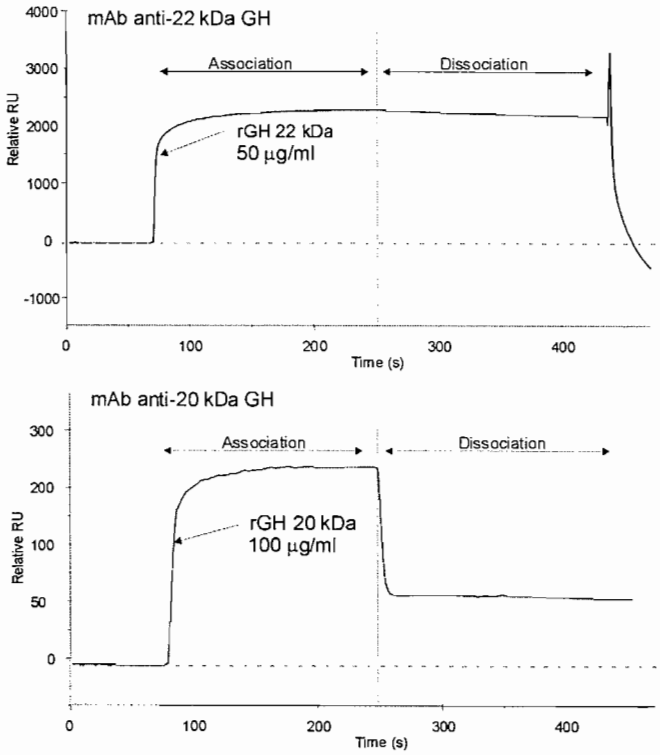

Figure 5. Sensorgrams corresponding to 22 kDa GH, interacting with A36020047P (upper panel) and 20 kDa GH, interacting with DO5 (lower panel).

Clearly, the surface properties of DO5 are significantly inferior (possibly due to immobilisation through the FAB domains) in comparison to those of A36020047P when handled under identical conditions. This example reinforces the need for extreme caution when differential immuno-sensing is to be employed.

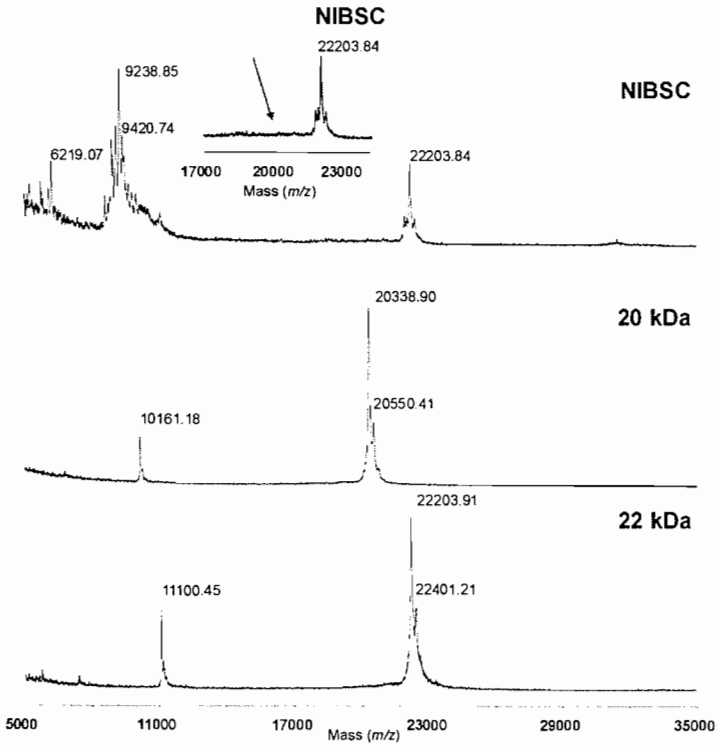

Figure 6. MALDI-TOF mass spectra corresponding to 22 kDa GH (lower panel), 20 kDa GH (central panel) and the pituitary extract from NIBSC (80/505, upper panel).

A different, but by no means less important, aspect of GH determination refers to the need for adequate standard material. In this sense the recombinant variants can be readily acquired from the pharmaceutical companies. However, an endogenous, pituitary derived standard is not readily available. The NIBSC 80/505 preparation could have been the material of choice for this purpose. However, mass spectrometric analyses in our laboratory of this particular preparation appear to indicate the opposite. A medium-intensity peak revealed the presence of the 22 kDa variant and no evidence could be found for the presence of the second most abundant (20 kDa) isoform (Figure 6). In addition, the most intense peaks were observed at *m/z* values below 10 kDa. Subsequent peptide-mapping analyses of individual SDS-PAGE-separated bands confirmed the identity of the 22 kDa isoform and indicated that the proteins below 10 kDa presumably correspond to degradation products of the 22 kDa variant. Recently we learned that the 22 kDa isoform is selectively enriched in the 80/505 preparation[11] (G. Baumann, Center for Endocrinology, Metabolism and Molecular Medicine, Northwestern University, Feinberg School of Medicine and Veterans Administration, Chicago Health Care System, Chicago, IL 60611, USA; personal communications at the USADA 3rd annual conference –Detection of Human Growth Hormone Abuse in Sport.).

CONCLUSIONS

Surface plasmon resonance appears to be an extremely useful tool for the evaluation of biomolecular interactions. As such it permits evaluating all surface properties of individual antibodies at the level of surface orientation, detection limits, linear range, and kinetic parameters under carefully controlled experimental settings. The technique does not require a secondary antibody nor labelled material in its detection principle. Thus, this simple one-to-one approach contributes significantly to the confidence with which immuno-sensing based protocols can be developed. In this context, the preliminary data have demonstrated that A36020047P represents an excellent candidate to monitor specifically the 22 kDa isoform. In contrast, the specific anti-20 kDa mAb DO5 exhibits relatively poor surface properties and requires a tailor-made approach to optimise its efficacy in such setting. The sensitivity of the technique currently still requires extensive sample handling in the case of urine but not in the case of plasma. Finally, a meticulous analysis of the standard pituitary derived GH preparation 80/505 raises reasonable doubts concerning the representativity of this material as the preferred reference preparation for GH assays.

Acknowledgments

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