

“A new integrated approach for the sensitive and unambiguous detection of prohibited protein substances in biofluids”

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Project Review:

Over the last decades, an increasing number of pharmaceutical peptides and proteins have entered the market. Unfortunately, protein drugs are also misused by athletes to illicitly enhance their performance. As these biopharmaceuticals often are highly similar to natural endogenous proteins, reliably tracing of protein doping products in urine or blood is very challenging. Current anti-doping methods can detect prohibited protein substances, but they do not provide unambiguous information on the molecular structure of the detected species, leaving room for errors. Therefore, advanced analytics such as liquid chromatography-mass spectrometry (LC-MS), have gained popularity in doping analysis. However, LC of intact protein molecules is troublesome, and so far few suitable LC-MS methodologies for distinguishing endogenous from manufactured proteins have been developed.

In this project, we will develop a highly selective and generic analytical platform for the unambiguous characterization of prohibited protein substances in biofluids, like urine and blood. To this end, capillary electrophoresis (CE) will be combined with high-resolution MS. In contrast to LC, CE can provide highly efficient separations of intact protein species. High-end MS detection will yield accurate molecular masses of the separated proteins and their isoforms. This way, CE-MS will allow consistent discrimination of banned protein species from endogenous proteins. In order to meet the requirement to measure very low protein concentrations, a novel ultrasensitive interface will be employed for coupling CE and MS. Moreover, for sample pretreatment, advanced affinity extraction and in-capillary preconcentration strategies will be exploited to achieve very low detection limits. Ultimate goal is to aid the fight against doping by delivering a new reliable method for high-throughput assessment of prohibited performance-enhancing proteins.

Results and Conclusions:

Results. Major efforts have been put in developing a robust and sensitive capillary electrophoresis method to allow hGH isoform separation and to live up to the requirements for doping analysis. Separation of the two main hGH isoforms can be achieved using a volatile alkaline background electrolyte (BGE). Evaporation of volatile BGE components, leading to severe method instability, could be circumvented by the use of a mineral oil overlay. The nature and order of conditioning showed to have a big impact on the migration time (MT) stability. After verifying several combinations, MT RSDs for the electroosmotic flow and analyte remained constant and were below 0.3% and 0.5%, respectively, for 48 consecutive injections.

High resolution mass spectrometry (MS) in combination with CE separation enabled structural characterization of endogenous hGH. Next to the differentiation of the 20 kDa and 22 kDa isoform, two known deamidated forms of asparagine were observed. Furthermore, two truncated forms, resulting from the loss of the first two amino

acids from the N-terminus, were distinguished. All these many other modifications are, most probably, formed during the long lifetime of this standard (over 30 years). Interestingly, the hGH isoform ratio observed in commercial human standards found with CE-MS (1:15-25) correlated well with reported values (~1:20). The CE method enabled for the first time to assess the affinity of commercial hGH antibodies on an individual isoform level. Careful optimization of the binding and elution steps revealed a good recovery for the 22 kDa isoform using one mAb. However, in all other cases low recovery was obtained. This raises the question whether current mAbs are able to accurately distinguish hGH isoform and should be used for sample clean-up and biochemical assays.

Conclusions. A simple and robust CE method has been developed that can separate the two main isoforms of hGH under MS compatible conditions. The hyphenation with MS detection ensures detailed characterization of hGH isoforms. It is the first time that unambiguous assignment of the 20 and 22 kDa variant was made possible showing a clear improvement over current methodologies. Unfortunately, the current commercial interfaces do not provide the sensitivity or robustness without further optimization. The developed CE method did indicate that using the available commercial antibodies raised against the two main isoforms of hGH do not show the same affinity.