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Review

Internal standards in the quantitative determination of protein biopharmaceuticals using liquid chromatography coupled to mass spectrometry

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ABSTRACT

Following the increase in development of protein biopharmaceuticals, there is a growing demand for the sensitive and reliable quantification of these proteins in complex biological matrices such as plasma and serum to support (pre)-clinical research. In this field, ligand binding assays (LBAs) are currently the standard analytical technique, but in recent years, there is a trend towards the use of liquid chromatography hyphenated with (tandem) mass spectrometry (LC–MS/MS). One of the reasons for this trend is the possibility to use internal standards to correct for analytical variability and thus improve the precision and accuracy of the results. In the LC–MS/MS bioanalysis of small molecules, internal standardization is quite straightforward: either a stable-isotope labeled (SIL) form of the analyte or a structural analogue is used. For the quantification of biopharmaceutical proteins, the situation is more complex. Since the protein of interest is digested to a mixture of peptides, one of which is subsequently used for quantification, there are more options for internal standardization. A SIL form or a structural analogue of either the intact protein or the signature peptide can be used. In addition, a modified form of the SIL-peptide internal standard, containing one or more cleavable groups is a possibility, and an internal standard can be generated during the analysis by using differential derivatization techniques. In this paper we provide an overview of the different options for internal standardization in the field of absolute targeted quantification of protein biopharmaceuticals using LC–MS/MS, based on literature from 2003 to 2011. The advantages and disadvantages of the different approaches are evaluated both with regard to the correction they provide for the variability of the different steps of the analysis and with regard to their generic availability. As most of the approaches used lead to acceptable results in terms of accuracy and precision, we conclude that there currently is no clear preferable method for internal standardization in the field of protein quantification by LC–MS/MS. It is essential, however, that any step in the analysis that is not covered by the internal standard chosen, should be carefully optimized and controlled.

Contents

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1. Introduction

In recent years, there has been a sharp increase in the development of macromolecular drugs, so-called biopharmaceuticals. In the period 2000–2009, 65 biopharmaceutical products received marketing approval from the US Food and Drug Administration (FDA), up from 39 in the 1990s and 13 in the 1980s [1]. Following this trend, analytical techniques to quantify biopharmaceuticals in complex biological matrices are continuously being developed and improved. The current standard method for quantifying proteins in biological matrices is based on ligand binding assays (LBAs). For years, no other analytical technique has been able to match the low detection limits of LBAs. Their excellent sensitivity and selectivity results from the use of an antibody raised against the protein of interest, or, in the case of the quantification of monoclonal antibodies (mAbs), the antigen, which very selectively extracts the analyte from the matrix, and significantly reduces the complexity of the sample.

LBAs require far lower investments in analytical equipment than chromatographic or mass spectrometric assays, have straightforward protocols and the 96- or 384-well plate they come in is truly a high-throughput format. However, when used for absolute quantitative determination of proteins some disadvantages arise [2]. Firstly, there is the time required to develop a new assay, typically some 4–6 months due to production and characterization of the antibodies and subsequent assay development and optimization. Secondly, there are analytical issues that can drastically influence results such as competition with endogenously generated anti-drug antibodies, non-specific binding and cross-reactivity which may remain undetected, since LBAs do not generate any chemical information about the analyte. Thirdly, most LBAs have a complex calibration model with a limited linear range. Finally, in LBAs, the use of an internal standard, correcting for these and other sources of variation, is technically not possible. Together with the fact that variation between different batches of antibodies is not uncommon, this may cause limited accuracy and precision, poor inter-laboratory reproducibility and significant discrepancies between products of different vendors.

Over the last few years it has been demonstrated that liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS), the golden standard for quantitative determination of low-molecular-weight drugs, can be a viable alternative to LBAs for the quantification of proteins [3,4]. Ongoing improvements in chromatography and mass spectrometry instrumentation have led to a situation where the sensitivity of LBAs is sometimes already rivaled by LC–MS/MS, although the approach is not free of its own problems and pitfalls [5]. One of the main strengths of the technique lies in the possibility to use internal standards that correct for different sources of analytical variability. Furthermore, analytical methods using this technique can be set up and validated in a relative short period of time of typically a few weeks.

A disadvantage of using LC–MS/MS in protein quantification is that proteins are incompatible with LC–MS/MS because their high molecular mass and size result in poor ionization efficiency, a signal that is distributed over a large number of charge states and very inefficient or non-existent collision-induced dissociation (CID). To resolve these problems, the protein needs to be digested to a mixture of smaller peptides, one of which is selected for quantification (the so-called signature or proteotypic peptide). The enzymatic digestion is, however, a potential source of variation and needs to be carefully controlled. In addition, selective extraction of the protein from the biological matrix is desirable to reach sufficient concentration sensitivity, which may be difficult to achieve without the use of immuno-affinity materials.

The transfer of protein analysis from the LBA to the LC–MS/MS platform is by no means straightforward. Compared to LBAs, the analytical approach is relatively complex, which makes the use of a proper internal standard essential. This paper describes and compares different approaches towards the use of internal standards in the field of quantitative bioanalysis of protein biopharmaceuticals. After a general discussion of the use of internal standards and the important step of protein digestion, an overview is given of different types of proteins and peptides as internal standards, the possibilities of differential derivatization to create internal standards and finally protein quantification without internal standards. Selected examples from the bioanalytical literature are used to compare and discuss the different approaches for internal standardization.

2. Protein quantification – general remarks

2.1. Internal standards

An internal standard is a compound that displays physical and chemical characteristics similar to that of the analyte of interest, but at the same time generates a response that can be distinguished from that of the analyte. Equal amounts of internal standard are added to all samples to be analyzed, and due to the similarity between the analyte and the internal standard, it is anticipated that their initial ratio does not change, because both suffer the same losses due to inefficiencies in extraction, digestion or ionization. Finally, both compounds are analyzed and the ratio of the measured signals is calculated. The internal standard thus corrects for variations in the analyte response caused by variability in the analytical procedure.

Mass spectrometric detectors for liquid chromatography have been widely used since the early 1990s. Before then, ultraviolet (UV) and fluorescence (FL) detectors were more common. An advantage of these detection systems over mass spectrometry is their stability. Good results in terms of accuracy and precision can be achieved, often even without the use of an internal standard, provided that sample handling steps are minimized and if needed, well optimized. When necessary, a compound displaying similar extraction and chromatographic characteristics as the analyte of interest can be used as internal standard to correct for instrumental variability. Due to the inability of these detectors to discriminate between the analyte and its internal standard, a chromatographic separation is required.

The reason why the much more expensive mass spectrometers rapidly became more popular is their enhanced sensitivity and selectivity compared to UV and FL detectors. Visible interferences from co-extracted matrix compounds or metabolites are much less common. However, when using mass spectrometry, the use