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A new reliable, transposable and cost-effective assay for absolute quantification of total plasmatic bevacizumab by LC–MS/MS in human plasma comparing two internal standard calibration approaches

Rachel Legeron[a,b,*,1], Fabien Xuereb[a,b,1], Stephane Chaignepain[c], Alain-Pierre Gadeau[b], Stephane Claverol[c], Jean-William Dupuy[c], Sarah Djabarouti[a,b], Thierry Couffinhal[b], Jean-Marie Schmitter[c], Domonique Breilh[a,b]

[a] Department of Pharmacy, Groupe hospitalier Sud, CHU de Bordeaux, Avenue de Magellan, 33604, Pessac, France
[b] Univ. Bordeaux, EPST, Biology of Cardiovascular Diseases, U1034, 33604, Pessac, France
[c] Univ. Bordeaux, EPST, CBMN, CGFB, UMR 5248, CNRS, 33076, Bordeaux Cedex, France

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ABSTRACT

The quantification of monoclonal antibodies (mAbs) such as bevacizumab, a recombinant humanized immunoglobulin G1 (hIgG1), in biological fluids, is an essential prerequisite to any pharmacokinetic preclinical and clinical study. To date, reference techniques used to quantify mAbs rely on enzyme-linked immunosorbent assay (ELISA) lacking specificity. Furthermore, the commercially available ELISA kit to quantify bevacizumab in human plasma only assesses the free fraction of the drug. However, the conditions of storage and analysis of plasma samples could alter the physiological equilibrium between the free, bound and partially bound forms of bevacizumab and this could result in over- or underestimation of drug concentration. We developed a new assay for absolute quantification of total fraction of bevacizumab by liquid chromatography tandem mass spectrometry (LC–MS/MS) basing identification and quantification of bevacizumab on two specific peptides. In this report we compare our assay with two internal standard (IS) calibration approaches: one using a different human mAb (Trastuzumab) and the other using a stable isotope labeled specific peptide. After enrichment by affinity chromatography on protein A and concentration by ultrafiltration, human plasma samples were proteolyzed by trypsin. Linearity was established from 12.5 to 500 µg/mL with an interday accuracy ranging from 101.7 to 110.6% and precision from 7.0% to 9.9%.

This study demonstrates the importance of the choice of the IS in quantifying bevacizumab in human plasma and highlights the difficulty of reaching a reliable proteolysis with a sufficient recovery. We developed a reliable and cost-effective LC–MS/MS method to quantify total plasmatic fraction of bevacizumab in human plasma. Through our development we proposed a generic methodology easily transposable to quantify all IgG1 subclass very useful for clinical pharmacokinetics studies.

1. Introduction

Monoclonal antibodies (mAbs) constitute a therapeutic class which is currently undergoing the fastest rate of development in various fields of pharmaceutical biotechnology. Among them is bevacizumab (Avastin®), a monoclonal antibody immunoglobulin G1 (IgG1) directed against the circulating vascular endothelial growth factor (VEGF) resulting in anti-angiogenic activity [1]. Bevacizumab has been approved for the treatment of metastatic colorectal, renal, pulmonary and ovarian cancers. However, although it significantly improves the therapeutic management of cancer, it is characterized by inter-individual variability in clinical response as seen with other mAbs whose causes remain poorly understood [2–4]. Several factors are currently being studied and among them the pharmacokinetic (PK) variability of mAbs could be a cause of response variability to treatment [2–4]. Evaluation of mAb concentrations in biological fluids is an essential prerequisite for the

* Corresponding author at: Department of Pharmacy, Groupe hospitalier Sud, CHU de Bordeaux, Avenue de Magellan, 33604, Pessac, France.
E-mail addresses: rachel.legeron@chu-bordeaux.fr (R. Legeron), fabien.xuereb@chu-bordeaux.fr (F. Xuereb), s.chaignepain@cbmn.u-bordeaux.fr (S. Chaignepain), alain.gadeau@inserm.fr (A.-P. Gadeau), stephane.claverol@u-bordeaux.fr (S. Claverol), jean-william.dupuy@u-bordeaux.fr (J.-W. Dupuy), sarah.djabarouti@chu-bordeaux.fr (S. Djabarouti), thierry.couffinhal@inserm.fr (T. Couffinhal), jm.schmitter@cbmn.u-bordeaux.fr (J.-M. Schmitter), rachel.legeron@chu-bordeaux.fr (D. Breilh).
[1] Co-first authors.

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determination of PK parameters to assess the relationship between clinical response and drug exposure. In this context, it is necessary to develop sufficiently sensitive and specific quantification methods to ensure accurate and reliable measurement of expected therapeutic concentrations in plasma, between 75 and 270 µg/mL for bevacizumab [3,4].

To date, reference techniques to identify and quantify mAbs rely on enzyme-linked immunosorbent assay (ELISA) methods due to their high sensitivity and recovery [5]. However, ELISA methods present important limits especially in terms of reliability. In fact, the specificity of ELISA techniques can be affected by cross-reactions with other molecules present in biological matrices especially when patients develop antibody against the mAbs and this can generate false negatives or false positives [6,7]. It has also been reported noteworthy differences in sensitivity and specificity between the various infliximab ELISA assay [8]. Furthermore, given their high sensitivity, linearity of response does not cover the full range of expected therapeutic plasma concentration. Samples therefore have to be diluted and this can lead to poorer accuracy and precision [6,9]. Moreover, bevacizumab can be found in three different forms in the human plasma: free, bound and partially bound to its plasmatic target (VEGF). The choice and the knowledge of the measured fraction are crucial to interpret the plasma concentrations and the resulting PK data. The commercial ELISA kit available for bevacizumab dosing in human plasma only assesses the free fraction of the drug. However, storage and analysis conditions of plasma samples alter the physiological equilibrium established *in vivo* between the different plasmatic forms of bevacizumab, resulting in over- or underestimation of the measured concentrations when quantification is conducted exclusively on free fraction [10]. An analytical method that does not disturb the physiological equilibrium would be ideal [11]. Taking into account how difficult it is to maintain the physiological equilibrium between the different plasmatic forms of bevacizumab, we resorted to an alternative strategy to study the total plasmatic fraction of the mAb [10]. This would overcome the variability associated with *ex vivo* disruption of the *in vivo* equilibrium and result in a more reliable PK analysis.

For this purpose, we developed an analytical strategy to quantify the total fraction of bevacizumab in human plasma. Liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS) is a well-known analytical alternative due to its high specificity, repeatability, precision and accuracy of the measurement [5,6,12–22]. The specificity of LC–MS/MS detection is achieved through the analysis in *selected reaction monitoring* (SRM) acquisition mode of a surrogate peptide obtained from the protein after enzymatic proteolysis [5,6,12,22]. Considering the protein complexity of human plasma, the plasma sample must be purified to separate the mAb from other abundant plasma proteins to improve sensitivity and reproducibility of the LC–MS/MS method. The choice of the purification method is crucial given that purification process conditions the blood plasma fraction (free, bound or total) of the drug quantified. In the literature, several approaches have been used to purify samples before the proteolysis step. The most recently used purification approach is immunoaffinity based on the recognition of therapeutic binding site. However, the total fraction of bevacizumab cannot be quantified by current approaches since the immunocapture is only based on the free binding site of the mAb [6,13]. Consequently the bound and partially bound fraction cannot be quantified. Another strategy consisting of precipitating plasma proteins with an organic solvent and thus conducting proteolysis on the protein pellet [12,21]. However, the samples obtained are not sufficiently rich in IgG and bevacizumab which damages the repeatability of the proteolysis. Another common approach involves removing the high-abundance plasma proteins from biological samples [19]. However, commercial protein depletion kits are unsuitable for measuring plasma concentrations of bevacizumab as they remove only albumin which is not sufficient, or they remove albumin and other abundant proteins including IgGs which cannot preserve mAb IgGs before proteolysis and LC–MS/MS analysis. Finally, given that the plasma sample pre-treatment steps and analysis by LC–MS/MS can generate variability, internal calibration approaches consisting of adding a fixed and known amount of an internal standard (IS) have been developed to correct these biases and improve accuracy of the assay [6,12–17,21]. Since MS detection of mAbs involves peptides derived from proteolysis of the protein, stable isotope labeled (SIL) surrogate peptides or a structural analogue of the intact protein are generally used as IS [6,13–15,21]. One of the widely described phenomena that could lead to variability in quantification, especially in electrospray ionisation (ESI) mode, is source ion suppression caused by matrix components [21,23]. The degree of ion suppression may vary greatly from one matrix to another and depends in particular on the chemical structure of the analyte [21,23]. Recent study of Osaki et al. consisting in MS-based method to quantify three mAb (including bevacizumab) with homologue mAb as IS provide that sequence similarity was more important than retention time for the surrogate peptide of IS [21]. Any ways it seems that these two factors are involved in the variability in the response. To correct this bias, the IS should have a similar chemical structure to the analyte and should present the same characteristics in terms of chromatography retention. For this, SIL surrogate peptides are often used as a way of correcting variability resulting from ion suppression phenomenon and chemical degradation of the signature peptide [19]. However, this approach does not take into account variations in the purification process on protein level and proteolysis. Thus, the use of an IS that would monitor all sample processing steps such as an analogue of the therapeutic protein of interest, emerged as another to consider [6,7,21,24,25].

Considering the increasing number of approved mAbs available on the market and the opening of the market to biosimilar, the need to conduct PK studies on mAbs is growing. However, the availability of a reliable analytical technique is a major pre-requisite for conducting preclinical and clinical PK studies. Now it common to say that LC–MS/MS is fully recognized as an analytical alternative to ELISA for mAbs quantification, it's necessary to develop a standardized LC–MS/MS quantification approach, easily transferable to other mAbs with minimal development, reliable and enough sensitive to perform PK studies [20]. For this purpose, we have developed and validated an analytical method for absolute quantification of total bevacizumab plasmatic fraction by an LC–MS/MS approach using protein A chromatography affinity followed by ultrafiltration to purify and concentrate samples. Considering that protein A contains four high-affinity binding sites interacting with the Fc region of IgG-class and that ultrafiltration device cut-off was 100 Kd, the benefit here is that the use of this purification process preserves the total fraction of bevacizumab in the sample. Due to the instability of "mAb-target" binding ex-vivo, determination of the total plasmatic form would be more reliable particularly for mAbs with a circulating target. In contrast to bevacizumab, for mAbs with target tissue, only the free fraction should be present in plasma. The combination of these two standard techniques for sample treatment allows transferring the assay to all IgG1 mAbs by using standard consumables. Over this development, we have compared the two most common strategies of internal calibration used, one using SIL surrogate peptide and the other using another therapeutic mAb IgG subclass (mAbIS), trastuzumab, to monitor the complete sample process. This comparison can be very helpful to make a choice between these two alternatives of IS when the stable isotope labeled mAb is not available which is currently the case due the complexity, time consuming and cost to product especially when a bio-similar is not available.

2. Materials and methods

2.1. Chemicals and reagents

Bevacizumab (Avastin®) and trastuzumab (Herceptin®) used as IS were both provided by Roche (Basel, Switzerland). Aqueous stock