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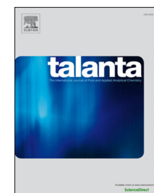

Simple and ultra-fast recognition and quantitation of compounded monoclonal antibodies: Application to flow injection analysis combined to UV spectroscopy and matching method

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ABSTRACT

Compounding of monoclonal antibody (mAbs) constantly increases in hospital. Quality control (QC) of the compounded mAbs based on quantification and identification is required to prevent potential errors and fast method is needed to manage outpatient chemotherapy administration. A simple and ultra-fast (less than 30 s) method using flow injection analysis associated to least square matching method issued from the analyzer software was performed and evaluated for the routine hospital QC of three compounded mAbs: bevacizumab, infliximab and rituximab. The method was evaluated through qualitative and quantitative parameters. Preliminary analysis of the UV absorption and second derivative spectra of the mAbs allowed us to adapt analytical conditions according to the therapeutic range of the mAbs. In terms of quantitative QC, linearity, accuracy and precision were assessed as specified in ICH guidelines. Very satisfactory recovery was achieved and the RSD (%) of the intermediate precision were less than 1.1%. Qualitative analytical parameters were also evaluated in terms of specificity, sensitivity and global precision through a matrix of confusion. Results showed to be concentration and mAbs dependant and excellent (100%) specificity and sensitivity were reached within specific concentration range. Finally, routine application on "real life" samples (n = 209) from different batch of the three mAbs complied with the specifications of the quality control *i.e.* excellent identification (100%) and ± 15% of targeting concentration belonging to the calibration range. The successful use of the combination of second derivative spectroscopy and partial least square matching method demonstrated the interest of FIA for the ultra-fast QC of mAbs after compounding using matching method.

1. Introduction

Therapeutic application of the monoclonal antibodies (mAbs) constantly grows in the hospital field [1–3]. The commercially available monoclonal antibodies, as well as other cytotoxic drugs, require compounding (*i.e.* dilution in appropriate physiological solution) prior to patient infusion. Considering the increasing number of patients to treat, compounding activity at the hospital is operated similarly to a large-scale production reaching over 35,000 preparations a year. According to the good manufacturing practices (GMP) and guidelines, quality control (QC) of the compounded mAbs is necessary to ensure the consistency of the preparation [4,5]. QC of biomolecules in the pharmaceutical field requires in-depth characterization including structure, heterogeneity, and bioactivity assessment, basically achieved with orthogonal and complex techniques [6,7] generally performed in research labs. Whereas, the main objectives of the routine QC at the hospital are to provide as fast as possible, i) a clear identification (*i.e.* recognition) of the monoclonal antibody as well as ii) the quantification of the preparation before release.

Various simple analytical methods have been proposed for the quantification and / or the identification of the mAbs including ELISA [8], capillary electrophoresis [9,10], LC-MS [11], HPLC [12], UV, Infrared or Raman spectroscopy [13,14]. However, most of these methods are time consuming, expensive, non-compatible with salines such as 0.9% NaCl solution or need sample pretreatment prior the analysis. If reverse phase liquid chromatography (RP-HPLC) is widely used for protein analysis [12], run time still remains non compatible with routine hospital QC of mAbs. Furthermore, adsorption remains one of the

Abbreviations: Bvz, bevacizumab; CS, control standard; ifx, infliximab; PCA, principal component analysis; QC, quality control; rtx, rituximab; SEN, sensitivity; SPE, specificity; trz, trastuzumab

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major issue of the mAbs in RP-HPLC analysis resulting in poor recovery and precision [15]. Besides, spectroscopy based methods are fast and simple. Indeed, Multispec® and QC prep+® (or QcRx®), two instruments using UV/IR and UV/Raman spectroscopy respectively, have been designed for the QC of drugs including mAbs [13]. For instance, QcRx® is designed with an automated filling of the 1 mL spectroscopic cell during the run. If quantification is easily achieved by spectroscopy, discrimination of mAbs remains difficult due to high interference of water and/or excipients of the therapeutic mAbs which present similar formulation [16]. Flow injection analysis (FIA) coupled to a diode array detector (DAD), also has the advantage to provide high throughput UV spectroscopy analysis [17] meeting the requirement for routine ultrafast QC of compounded drugs [18]. The method is based on a flow stream that directly carries a small volume of sample to the detector. FIA has already been used in pharmaceutical fields for the analysis of chemicals [19–21] and proteins [22,23]. However, UV absorption spectra remain limited to provide qualitative information. For instance, excipients from the formulation may influence the absorption by partial spectral overlapping. In that context, derivative spectroscopy appears as a convenient approach to overwhelm the matrix effect (*e.g.* excipients). Indeed, common excipients used in mAbs formulation gathered polysorbates, histidine, carbohydrate [24] that absorb at low wavelength typically under 230 nm. Moreover, the higher the derivative order, the lower their spectroscopic contribution. On the other hand, many studies reported the interest of the aromatics residues of proteins for structure characterization or probing purpose in the spectral range 245 – 300 nm [25–28]. Targeting the analysis on the aromatic amino acids of the mAbs, phenylalanine (phe), tyrosine (tyr) and tryptophan (trp), within the spectral range from 245 nm to 300 nm, excellent discrimination of four mAbs (100% specificity and sensitivity) has been reported using second derivative UV spectroscopy and partial least square discriminant analysis (PLS-DA) in a previous study [14]. In this study, algorithms of function of classification have been built by PLS-DA from the data of the second derivative spectra in the range 265–300 nm. However, intensive analysis by multivariate analysis involving chemometric software has been performed and the implementation of these algorithms in computer software of HPLC instruments remains difficult. Most of these modern HPLC softwares provide spectral searching algorithms for matching methods necessary for spectral comparison or identification. A few algorithms have been built to compare spectra such as correlation coefficient [29], area of overlap [30] or spectral difference method [31]. The exact algorithm performed for the match processing in HPLC software is often unknown by the users. Nonetheless, the basic process includes the comparison of an unknown spectrum against the spectra of the library source and a Euclidian distance or similarity index can be measured between the spectra. However, quality, complexity, shape and high similarity of the spectra may complicate their successful matching. The match depends therefore on the ability of the algorithm to accurately recognize subtle spectral differences between samples.

The aim of the present study was to propose a QC using FIA coupled to a DAD and a least square matching method for both the quantification and the discrimination of the mAbs. For this, three well represented mAbs *i.e.* rituximab, infliximab and bevacizumab, have been selected for their high structural similarity, their different therapeutic range as well as their increasing use in hospital.

2. Materials and methods

2.1. Chemicals

Ultrapure water was provided by a milliQ purification station from Millipore (Bedford, USA). Polyolefin freeflex bags of 0.9% sodium chloride were from Fresenius Kabi (Fresenius Kabi, France).

2.2. Sample and standard preparations

Bevacizumab (Avastin®, 25 mg mL^{-1}), trastuzumab (Herceptin®, 150 mg) and rituximab (Mabthera®, 10 mg mL^{-1}) were purchased from Roche (Switzerland). Infliximab (Inflectra®, 100 mg) was from Hospira Ltd (the United Kingdom). Sterile Polyolefin Freeflex bags of 0.9% NaCl were from Fresenius Kabi (Fresenius Kabi, France). Infliximab and trastuzumab were first diluted in sterile water for infusion to reach a 10 mg mL^{-1} concentration as required by the manufacturer guidelines. Each monoclonal antibody was diluted with saline (0.9% NaCl) under aseptic conditions yielding to concentrations ranging from 1 to 20 mg mL^{-1}; 0.5–5 mg mL^{-1}, and 0.5–1.9 mg mL^{-1} for bevacizumab (bvz), rituximab (rtx) and infliximab (ifx) respectively. Appropriate low/medium/high standard solutions were prepared at 3, 12, 17 mg mL^{-1}; 0.8, 3, 4.5 mg mL^{-1} and 0.7, 1.4, 1.8 mg mL^{-1} for bvz, rtx and ifx respectively. Each respective mAbs were from the same batch. Fresh solutions of mAbs were immediately analyzed. Samples from infusion bags of patients *i.e.* "real life" samples were prepared from different batches, under cytotoxic safety cabinet.

2.3. Analytical QC method

The analytical QC of the compounded drugs is based on distinct analytical conditions depending of each tested drug. Such procedure allows the adjustment of quantitative analysis to the therapeutic range of the drugs (distinct calibration range and injection volume). Consequently, proper injection volume was applied for the determination of each monoclonal antibody, as their calibration range was different (1–20 mg mL^{-1}, 0.5–5 mg mL^{-1} and 0.5–1.9 mg mL^{-1} for bvz, rtx and iflx respectively). Fig. 1 describes the workflow process of the analysis of the mAbs. Identification as well as the quantification of the mAbs is carried out at the same time from the absorbance spectrum.

Flow injection analysis (FIA) was performed using HPLC Dionex Ultimate 3000® (Thermo Scientific, USA) hardware including a diode array detector. 0.9% NaCl sterile solution from Fresenius Kabi (Fresenius Kabi, France) was used as carrier stream. The flow rate was set at 0.5 mL min^{-1}. The volume of injection applied for rtx, bvz and ifx was 1, 2 and 10 μL respectively, according to their calibration range. The quartz cell detector had an optical path length of 10 mm. The acquisition frequency was 5 Hz and the spectral resolution was 1 nm. The system was entirely controlled by Chromeleon® software analyzer (Thermo Scientific, USA) providing analysis and collection of the data. Spectral acquisition was performed from 190 to 400 nm and the signal of the FIA was evaluated by the area under the curve at 280 nm to estimate the concentration of the analyzed sample (Fig. 1).

2.3.1. Spectral data handling

2.3.1.1. Quantitative validation of the analytical conditions. A five levels linear calibration was fitted by least square regression from the area under the curve (AUC) of the FIA signal as a function of the sample concentration at a detection set at 280 nm. The y-intercept, coefficient of determination R^2, and the slope of the linear regression, have been evaluated. The accuracy of the method was assessed from nine determinations over three concentration levels of the control standards to calculate the recovery and relative error. For the precision, intraday and inter-day precisions have been evaluated. The repeatability and the inter-day precision were assessed from six consecutive injections in one day (n = 6) and three consecutive injections in three days (n = 9) respectively. ANOVA was applied to evaluate the precision.

2.3.1.2. Qualitative validation of the methods. According to Fig. 1, the second-order spectra resulted from Dionex Chromeleon® 7.2 software (Thermoscientific, USA). The second derivative spectra were calculated by adaptive Savitzky-Golay from the corresponding spectra of absorption (190–400 nm) obtained with the FIA coupled to the DAD

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