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**Importance of vial shape and type on the reproducibility of size exclusion
chromatography measurement of monoclonal antibodies**

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ABSTRACT

The goal of this work is to understand the phenomenon behind the poor injection repeatability often observed with protein biopharmaceuticals. All the measurements were carried out in size exclusion chromatography (SEC) mode, using fifteen commercially available therapeutic monoclonal antibodies (mAbs). First of all, we proved that the variation of peak areas between consecutive injections was much more critical with highly concentrated mAb samples (up to 80 mg/mL), while the SEC measurements of commercial mAbs having concentrations below 5 mg/mL were reliable. Second, we emphasized that the shapes, volumes and materials of the sample vial insert also contribute to the change of peak areas observed during consecutive injections. In this study, six different insert models were studied and the most critical were the ones possessing the narrowest conical shape at the bottom. Furthermore, the homogenization of samples (with pipette mixing rather than vortex) prior to analysis was of great interest and allows a significant improvement in injection repeatability. Finally, because the on-wall (on-insert) adsorption of mAbs reaches its

equilibrium in around 50 minutes, it is advised to add a specified residence time prior to injection to achieve repeatable injection.

Keywords:

monoclonal antibody, protein biopharmaceuticals, size exclusion chromatography, vial, insert, injection precision

1. Introduction

Reproducible quantitation of proteins by liquid chromatography (LC) is known to be more critical than for small molecules [1,2,3]. The reason of data inaccuracy with this type of analyte is possibly a sum of several potential contributions, such as protein adsorption to the system (injector, tubing, detector), adsorption at the surface of the stationary phase, adsorption to the wall of sample vial, difficulties with injection or homogenization of highly viscous protein solutions, and perhaps also the sedimentation of poorly soluble proteins in the vial. All these different contributions are detailed below.

The inertness (bio-inertness) of systems applied for biopharmaceutical analysis is crucial. When working with protein samples, it is suggested to avoid the use of polyether ether ketone (PEEK) for connection tubing and injection needle [4,5]. PEEK is indeed a hydrophobic material that can cause strong protein adsorption. Inert materials such as titanium, stainless steel or PEEK-Sil (fused silica inside, PEEK outside) are preferable, though fused silica and stainless steel do not completely eliminate protein adsorption [6]. Agilent Technologies introduced the 1260 Infinity Bio-inert HPLC system, which was dedicated to biopharmaceuticals characterization. This system is iron- and steel-free in solvent delivery, and the sample-contacting surface is completely metal-free, minimizing undesired surface interactions. Waters Corporation (Milford, USA) also launched an inert UHPLC system (ACQUITY UPLC® H-Class Bio System) that features an inert flow path. Other vendors also offer bioinert LC systems, such as the Shimadzu LC-10Ai HPLC system

or the Dionex Ultimate 3000 system. Finally, Jasco recommends the use of some bioinert LC modules for the LC- 2000 system.

Protein adsorption to column packing material is also a well-known issue [2]. It has been shown that therapeutic monoclonal antibodies (mAbs) are especially prone to adsorb at the surface of the stationary phase in reversed phase (RPLC) mode [7,8,9]. A systematic study demonstrated that IgG1 and IgG2 type mAbs (i.e. rituximab, panitumumab, and bevacizumab) have significantly higher adsorption rate than model proteins of the same size [7]. In addition, some significant recovery differences were highlighted between several RP columns (silica based and BEH hybrid). This behavior is likely to be related to the difference in the contribution of strong secondary interactions (i.e., ionic and hydrogen bonding). These observations have suggested that mAbs adsorption is linked to secondary interactions rather than pure hydrophobic interaction mechanism. Adsorption rate also depends on the size of mAbs fragments. Indeed, the lowest recovery has always been observed for the intact mAbs of around 150 kDa, while better results were observed for the fragments of 25-50 kDa. A solution to reduce on-column adsorption phenomenon is the use of high mobile phase temperature (up to 80–90 °C) [7,9]. Indeed, the secondary ionic interaction kinetics (adsorption/ desorption) can significantly be improved, leading to substantial increase in recoveries of mAbs and their fragments. However, at such elevated temperature, the risk of thermal degradation should also be considered and the solute residence time inside the column should be limited. An alternative solution to decrease on-column adsorption is to add a small amount of an ancillary solvent (1 to 3% n-butanol) to the mobile phase. It has been demonstrated that the hydroxyl group of n-butanol probably interacts with the water layer adsorbed on the surface, to “shield” silanols [10]. Please note that protein adsorption to the column inlet frit is also possible [1].

Beside on-column adsorption, another important issue is the protein adsorption on the wall of sample vials. Proteins are surface active molecules, which undergo non-specific adsorption when getting in contact with surfaces such as packing-, or storing materials [11]. This process could be critical as it may cause a loss of protein content or aggregation. During the

adsorption, some proteins undergo shape changes (conformational changes). The area on the adsorbent surface (vial wall) that is occupied by the protein is often called “the footprint”. The footprint usually increases with residence time that is referred to as protein spreading process. Protein adsorption is usually partially reversible since proteins undergo some structural changes due to adsorption and are therefore attached with more and more segments to the surface (more interactive groups) [12,13]. The adsorption kinetics and spreading strongly depends on the protein concentration [14]. At high concentration, the surface is occupied in a shorter time sequence and the time for spreading is then shorter. This results in a smaller average footprint, but the adsorbed concentration will be higher. Other parameters such as solvent pH and ionic strength also impact protein adsorption. The influence of pH and ionic strength as formulation parameters on IgG adsorption to vials has recently been studied [15]. The vial adsorption phenomenon is observed in many different types of materials such as glass, siliconized glass or plastic vials [12]. Very recently, polyglycerol based coatings were used to reduce and control non-specific protein adsorption in sample vials [11].

Another possible source of LC measurements errors is related to the viscosity of highly concentrated therapeutic protein solutions (especially mAbs) [16,17]. The viscosity of protein solutions strongly depends on protein size and concentration and also on the amount of added salts (excipients). Therapeutic mAbs (150 kDa proteins) are typically commercialized in 2 - 50 mg/mL solutions and also contain salts and non-ionic surfactants in relatively high amount (few mg/mL of sodium chloride, sodium phosphate, sodium citrate and 0.1-1 mg/mL polysorbate). Viscosity values between 5 and 500 mPas are often reported for therapeutic mAb solutions [17,18], and such elevated sample viscosity may be problematic for some autosamplers. If the syringe rate is too fast, viscous liquids may not pass through small openings quickly enough, causing volumetric errors. This might be prevented by, for instance, a brief (1-3 sec) delay after sample injection [1].

Quantitative variation of around 2-3% for duplicate injections of the same sample usually indicates an HPLC injecting system problem, such as air bubbles in the syringe or the high

viscosity of the sample. Air bubbles can be caused by a restricted sample delivery tube, by the needle, or by too-rapid syringe movements [1].

Huebner and Bietz reported important reproducibility issues in RPLC and SEC measurements of wheat proteins [1]. When performing duplicate injections, the second sets of results often differed significantly from the first one. Pharmaceutical industrial laboratories are also continuously facing with this reproducibility issue, but the number of publications on this topic is still limited.

The purpose of this study was to investigate and understand the phenomenon behind the protein injection repeatability issues. For this purpose, some of the most critical protein biopharmaceuticals, namely mAbs, were used as test solutes. Fifteen different mAbs approved by the Food and Drug Administration (FDA) and the European Medicine Agency (EMA) were employed as case studies. SEC chromatographic mode was selected since the main advantage of this chromatographic mode is the mild mobile phase conditions that permit the analysis of proteins with minimal impact on the conformational structure and local environment [19]. On-column adsorption is also negligible in SEC mode, therefore we can avoid disturbance related to this feature. **The inertness of our UHPLC system has already been proven earlier** [7,8]. A bio-inert needle is used and the absence of on-system protein (mAb) adsorption was verified. This way we could focus on processes that occur exclusively in the sample vials inserts and/or related to sample injection.

2. Experimental

2.1. Chemicals, Columns and Vials

Water was obtained with a Milli-Q Purification System from Millipore (Bedford, MA, USA). Disodium hydrogen phosphate and sodium chloride were purchased from Sigma-Aldrich (Buchs, Switzerland). Hydrochloric acid solution at 37% concentration was also purchased from Sigma-Aldrich.

FDA and EMA approved therapeutic IgG monoclonal antibodies including adalimumab, belimumab, bevacizumab, cetuximab, denosumab, entanercept, infliximab, ipilimumab, natalizumab, ofatumimab, palivizumab, panitumumab, pertuzumab, rituximab, trastuzumab were kindly provided by the Center of Immunology Pierre Fabre (Saint-Julien en Genevois, France).

Yarra 3 μm SEC-3000, 300 x 4.6 mm column was purchased from Phenomenex (Torrance, CA, USA).

Six different types of sample inserts/vials possessing different volumes, shapes and materials were employed in this study. 150 μL conical glass insert (31 x 5 mm, tip 15 mm), 200 μL conical glass insert (31 x 5 mm, tip 9 mm), 250 μL conical glass-silanized insert (31 x 6 mm, tip 15), 250 μL conical glass-silanized insert with plastic spring (29 x 5.7 mm) and 300 μL short thread vial (32 x 11.6 mm) were purchased from BGB Analytik Vertrieb GmbH (Rheinfelden, Germany). A 300 μL conical glass insert (31 x 6 mm, tip 12 mm) was obtained from Schmidlin-lab (Neuheim, Switzerland). The different types of vial inserts and their properties were summarized in Figure 1.

2.2. Equipment and software

All the experiments were performed using a Waters Acquity UPLC™ system equipped with a binary solvent delivery pump, an autosampler and fluorescence detector (FL). The Waters Acquity system included a 5 μL sample loop and a 2 μL FL flow-cell. The loop is directly connected to the injection switching valve (no needle seat capillary). The connection tube between the injector and column inlet was 0.13 mm I.D. and 250 mm long (passive preheating included), and the capillary located between the column and detector was 0.10 mm I.D. and 150 mm long. The overall extra-column volume (V_{ext}) is about 14 μL as measured from the injection seat of the auto-sampler to the detector cell. The measured dwell volume is around 100 μL . Data acquisition and instrument control was performed by Empower Pro 3 Software (Waters). Calculation and data transferring was achieved by using Excel templates.

The mobile phase pH was measured using a SevenMulti S40 pH meter (Mettler Toledo, Greifensee, Switzerland).

2.3. Apparatus and methodology

2.3.1. Mobile phase composition and sample preparation

For the SEC measurements, the mobile phase consisted of aqueous salt solution containing 0.2 M sodium chloride and 0.1 M disodium hydrogen phosphate. The mobile phase pH was adjusted to pH = 6.8 by adding 1M HCl solution.

For preliminary measurements, the mAbs were injected directly from their parent solution (formulation) without dilution. The concentration of parent mAb formulations varied between 2 and 80 mg/mL.

For a systematic comparative study, the mAb samples were diluted in water to 1.5 mg/mL and stored in refrigerator at 4°C. Samples were injected using vial inserts of different volumes and shapes (see in § 2.1.). Perhaps it would have been better to dilute the samples with their own formulations, however this is hardly feasible. The different mAbs are formulated in different solvents and in many cases their exact composition is not available.

2.3.2. SEC chromatographic conditions

To elute mAbs from the 300 x 4.6 mm Phenomenex Yarra 3 µm SEC-3000 column, the mobile phase consisted of 0.2 M sodium chloride and 0.1 M disodium hydrogen phosphate (pH = 6.8). The flow rate was set to 0.3 mL/min, and temperature was set to 25°C. Detection has been carried out using fluorescence detection (λ_{ex} : 280 nm, λ_{em} : 360 nm, 20 Hz sampling rate). A volume of 2 µL was injected. The analysis time was 17 minutes.

2.3.3. Preliminary measurements, testing injection repeatability

First, the repeatability of consecutive injections was studied for all the fifteen mAb samples by injecting them from their parent formulation (2 – 80 mg/mL). Four consecutive injections were carried out and the change of observed peak areas was recorded. Plots of peak area

percentages were constructed and the differences of repeated peak areas were plotted as a function of mAb concentration.

2.3.4. Comparing different vial inserts and sample residence time

For studying the impact of time spent in vial insert (residence time) and the impact of sample homogenization (mixing), two different series (A and B) of experiments have been performed by using six different insert types and injecting adalimumab as model compound.

These two series were analysed at different days. When mixing was required, it was carried out by “pipette mixing” (3 times) rather than by vortex. Based on our preliminary results, vortex mixing was indeed not appropriate for very narrow inserts.

For the “A” series, samples were injected on the first day with mixing, then on the 4th day without sample mixing, on the 10th day with sample mixing and finally on the 15th day without sample mixing. For the “B” series, samples were injected on the first day without sample mixing, then on the 4th day with sample mixing, on the 10th day without sample mixing and finally on the 15th day without sample mixing. Triplicate injections were performed from each vial at each time.

As a control, the same experiments were performed in one 2 mL normal sample vial (without insert) and with sample mixing before injection.

Peak areas of the first and third injections (of the consecutive triplicates) were compared by the mean of their relative differences. Plots of peak area relative difference vs. residence time were constructed for each vial and for the two series of experiments.

2.3.5. Studying the time dependence on peak area (for system suitability)

Guidelines suggest performing at least five consecutive injections of the same sample to evaluate the system repeatability (precision) [20]. For this purpose - using the same conditions as in section 2.3.2 - five consecutive injections were performed from the “worst case” vial insert (type “C” in Figure 1). Plots of peak areas vs. elapsed time were constructed for the intact (monomer) and for a truncate adalimumab species.

2.3.6. Studying the impact of needle offset

One can assume that repeatability may depend on the position of the needle along the insert. If the repeatability issue is related to protein adsorption on the insert wall, then indeed the needle offset position can play a role since the ratio of specific wall area and protein mass changes along the vertical position of a conical vial insert. In addition, sometimes sedimentation could also be assumed. By injecting the same sample from different vertical positions, conclusions on possible protein sedimentation can be easily drawn. For the above mentioned purposes, five consecutive injections were made from the same vial ("worst case" type "C" insert) with two different needle offsets: at 2 mm position from the bottom and at 10 mm from the bottom. Plots of peak area percentages vs. elapsed time were prepared for the two different needle offsets.

3. Results and discussion

In most cases, vial inserts are used for the HPLC analysis of mAbs for several reasons: 1) only a limited sample amount is available, 2) samples are not always stable in vials (temperature, homogenization, change of contact surfaces when transferring the samples ...) and 3) mAb samples are quite expensive. For all of these reasons, the lowest amount is generally transferred to sample vials and used for analysis. However, chromatographers have to be careful since several different types of HPLC vial inserts are available on the market and there are significant differences in their shapes, volumes and materials. Therefore, the different insert types can have diverse impacts on processes which can occur in vials - such as protein adsorption or sedimentation. In the past, we have often observed issues with the reproducibility of consecutive injections of mAbs in various chromatographic modes. Then, we were interested to evaluate the impact of vial inserts on consecutive injections. For this purpose, six different types of widely used inserts were tested in this systematic study.

3.1. Injection repeatability and parent mAb formulation concentration

In a first instance, four consecutive injections of fifteen different mAbs were performed in SEC conditions. For these preliminary experiments, the most commonly used vial insert was applied. This one is a 300 μ L (31 x 6 mm) conical insert, quite narrow at the bottom ("C" type insert on Figure 1). The samples were directly injected from the parent mAb formulations. The samples possessed a wide range of concentrations (2 – 80 mg/mL). On-wall adsorption should be related to protein concentration, with this first series of experiments, the relation between mAb concentration and possible on-wall adsorption could be observed.

Figure 2 shows the change in observed peak area (compared to the first injection) during four injections. Based on this figure, only two mAbs (ipilimumab and cetuximab) showed repeatable injections (constant peak area), and another one (natalizumab) showed only a slight increase of peak area. The other twelve mAbs showed an obvious decrease in peak area with a similar trend. Typically, only 50 – 80 % of the peak area could be observed (recovered) in the second injection and only the 20 – 70 % of the original peak area was observed in the third injection. An interesting observation is that the peak areas remained constant after the third injection. (The fourth injection resulted in very similar peak areas as the third one.) It suggests that the on-wall (on-insert) adsorption of mAbs - if it is the main reason of the peak area decrease - is a relatively fast process and reaches its equilibrium in around 50 minutes (3x17 minutes analysis).

Among the fifteen mAbs, the two mAbs that showed the most repeatable injection - cetuximab and ipilimumab - possessed the lowest concentration (2.2 and 5 mg/mL, respectively). On Figure 3, the relative decrease of peak area between the first and second injections for the fifteen model mAbs was reported as a function of the mAbs concentration. If we disregard the results obtained with natalizumab (that showed a unique behavior), a nice relationship was observed between the mAb concentration and peak area decrease. This result suggests the existence of on-wall adsorption, since a higher amount of adsorbed mAb is expected at higher concentration. For some reason, natalizumab showed a different behavior. It possessed more or less a constant peak area (slight increase), despite its parent

concentration was 20 mg/mL. We have to mention here that adsorption also depends on the formulation pH and on the excipients. Maybe, the composition of natalizumab solution was quite different from the other therapeutic mAbs.

All these results suggest the existence of mAb adsorption on the insert wall. In most cases, a significant decrease of peak areas (up to 40%) can be observed in consecutive injections, except for the less concentrated mAbs.

3.2. The impact of insert type, residence time and sample homogenization

As above mentioned, several types of vial inserts are commercially available and their shapes, volumes and materials certainly contribute to the change of peak areas observed during consecutive injections. In this study, six different insert models were studied. Triplicate injections were performed on different days, using samples that were homogenized in different time intervals. **In this way, the impact of the insert itself could be studied, as well as the residence time available for adsorption (the time that the mAb spent in the vial stored in the sample rack in vertical position at 4 °C) or sedimentation. This protocol of experiments also enabled us to investigate the impact** of homogenization (that can indicate the existence of reversible or irreversible adsorption).

Figure 4 illustrates the decrease of peak areas obtained in the third injection relative to the first injection for the different vials and time intervals. The yellow boxes show the experiments corresponding to the samples homogenized for the two different experimental series. By comparing the peak areas decrease observed on the first day between homogenized (Figure 4A) and non-homogenized (Figure 4B) samples, it can be clearly seen that repeatable injection was feasible with homogenized samples with any insert type. The maximum deviation was only around 2 %. In contrast, when samples were not homogenized, a significant decrease in peak areas was observed with the A and C type inserts. This result suggests that: 1) on-wall adsorption rate is high for these two insert types and, 2) in a short time interval, the adsorption is reversible, since after sample homogenization, the injection becomes repeatable.

When comparing the data observed on the fourth day, important differences were also observed between these two series of experiments. Without homogenization, the A and C type inserts showed again some serious deviations (probably adsorption related to the four days long storage). However, when the samples were homogenized, the differences in peak areas were much lower (below 5 %). Nevertheless, the decrease of consecutively injected peak areas of homogenized samples were considerably higher on the fourth day than on the first day (2-4 % vs. 0-2 %) and absolute peak areas were lower on the fourth day. These results now suggest some non-reversible on-wall adsorption, probably related to the long-term storage in the inserts. Protein adsorption is generally reversible within a short time interval but, since proteins can undergo some structural changes during long-term storage, they attached with more and more segments to the surface. It may result in stronger interactions between the proteins and insert walls. According to the results reported in figure 4, it seems that this interaction is strong enough to resist a “mild” homogenization.

After ten days long storage, the deviation between homogenized and non-homogenized samples becomes even smaller. Finally, after 15 days, the deviations in consecutive peak areas were below 5 % for both the homogenized and non-homogenized samples, while peak areas scatter randomly (both a slight increase and decrease of peak areas were observed). This suggests that after 15 days long storage, the non-reversible in-vial adsorption has probably reached the equilibrium state.

This experimental setup allows assuming a two steps on-vial adsorption process. The first step occurs in a short residence time interval and is probably a reversible type adsorption. But after longer storage time (few days), the adsorbed mAbs may undergo some conformational changes and evolve into a stronger interaction with the vial wall. This second step of the process seems to be irreversible (at least resistant to homogenization or sample mixing).

On the other hand, it is also clear that the adsorption rate is particularly critical with the A and C inserts. These two insert types should be avoided for the LC analysis of mAb samples. They possess the narrowest conical shape at the bottom. So, it may happen that not only

adsorption is critical but also probably the homogenizability of the sample in such a narrow volume is limited.

Figure 5 shows a head-to-head comparison of data observed with a reference vial (without insert) and with the A type insert obtained in the two series of experiments. With the reference vial, constant peak areas were observed during 15 days long storage, while with the insert A, obvious decrease of absolute peak area was observed as a function of the residence time (in both types of homogenization procedures).

3.3. System suitability, addition of residence time prior to injection

Based on the above described results, it may be difficult to set system suitability criteria for injection repeatability. The best way would be to use standard vials without insert, but it is hardly feasible in practice due to the limitation of available precious mAb samples. As we have seen in section 3.1., a possible solution can be to wait a reasonable time (e.g. 50 minutes) after transferring the sample into the insert prior to injection, since the first step of adsorption process seems to reach its equilibrium within this time scale.

Figure 6 shows the observed peak area of native adalimumab ($t_{\text{elution}} = 9.9$ min) and its aggregate form (dimer, $t_{\text{elution}} = 8.7$ min) vs. elapsed time. As expected, both the native and aggregated adalimumab showed reasonable repeatability with a relative standard deviation (RSD) of 9.6 % and 4.3 %, respectively, during the five consecutive injections. No obvious trend can be observed, peak areas seem to scatter randomly.

Figure 7 presents the overlapped chromatograms of five consecutive injections of adalimumab with inset, zooming on the dimer peak. It is important to mention that adsorption-diffusion rate strongly depends on concentration. Moreover, it is probably a competitive time-dependent adsorption mechanism [21,22]. This is why we probably see higher deviation in peak area for the native form which is present in much higher concentration than the dimer.

The reported data prove that repeatable consecutive injections are hardly feasible in SEC. The addition of a short residence time prior to injection (e.g. sample stored in the insert for 50

minutes) helps to achieve more repeatable injection but repeatability is still not comparable to small molecules. Generally, the quantitation of aggregates and fragments in SEC is based on peak area %. The peak area % of low concentrated species may show some time dependency relative to the main monomer peak.

3.4. The impact of needle offset on data repeatability

A clear distinction between adsorption and possible sedimentation of poorly soluble proteins can be made if the injection needle position is changed. Indeed, the specific insert surface area per protein mass is much larger close to the bottom of the vial when using narrow conical inserts (A and C types). In addition, if sedimentation occurs, different peak areas should be observed when injecting from the bottom or from a position closer to the middle of the insert.

Five consecutive injections were performed from the same vial (“worst case” type C insert) with two different needle offsets: at 2 mm and a 10 mm position from the bottom. Figure 8 shows the obtained peak areas vs. the time spent by the mAb in the insert. The absolute decrease in peak area observed in 80 minutes was very similar, but the trend was somewhat different. A faster initial adsorption rate was observed at 2 mm vs. 10 mm offset position. It is in agreement with the larger specific insert area available for adsorption close to the vial bottom. On the other hand, these results do not confirm the existence of sedimentation (at least within the studied time scale). If sedimentation would be important, significantly larger peak areas should be observed with a needle position close to the vial bottom compared to the higher needle position.

Adalimumab sedimentation speed (v_t) in its parent solvent was estimated based on the Stokes-law:

$$v_t = \frac{g * d^2 * (\rho_{mAb} - \rho_{solvent})}{18 * \mu} \quad (1)$$

with density: $\rho_{\text{mab}}=1280 \text{ kg/m}^3$, $\rho_{\text{solvent}}= 1230 \text{ kg/m}^3$, gravity: $g=9.81 \text{ m/s}^2$, viscosity: $\mu=1.52 \text{ mPas}$ at $25 \text{ }^\circ\text{C}$, mAb diameter: $d=10^{-7} \text{ m}$. It was found that 0.5 cm sedimentation should take around 65 days.

To conclude, needle offset may have an impact on the observed peak area especially when using narrow conical inserts, but sedimentation seems to be negligible within the time scale of typical SEC analysis.

Figure 9 summarizes the possible processes and parameters that can affect the observed chromatographic peak area of mAbs and probably play a role in the repeatability of consecutive injections.

4. Conclusion

In this study, the phenomenon behind the poor injection repeatability of biopharmaceuticals was investigated. The measurements were performed in SEC, using fifteen different therapeutic mAbs. In a first instance, the commercial formulations of mAbs were tested, without dilution. Under these conditions, we were able to clearly demonstrate that the variation of peak areas between consecutive injections was particularly high for the most concentrated samples (up to 80 mg/mL), while the repeatability obtained with the two mAbs at concentrations below 5 mg/mL (ipilimumab and cetuximab) remained acceptable. This poor repeatability was clearly related to on-wall (on-insert) adsorption of mAbs. Because the adsorption equilibrium was reached after around 50 minutes, repeatable peak areas can be obtained when adding a short residence time of less than 1 hour prior to injection.

Various sample vial inserts having different shapes, volumes and materials were tested and the adsorption can be minimized with an adequate insert model. Indeed, the most critical inserts were the ones possessing the narrowest conical shape at the bottom. Another good way to improve peak areas repeatability is to homogenize the sample (with pipette mixing rather than vortex) prior to analysis.

By performing experiments over a 15 days period, we showed that a two steps on-vial adsorption process occurred. The first step takes place in a short residence time and is

probably a reversible adsorption, while the second step occurs after long term storage and since the adsorbed mAbs may undergo some conformational changes and evolve into a stronger interaction with the vial wall, this process seems to be irreversible (at least resist to homogenization or sample mixing). Finally, by modifying the injection needle position, the possible sedimentation of poorly soluble proteins can be evaluated. Our results do not confirm the existence of sedimentation (at least within the studied time scale).

5. Acknowledgments

The authors acknowledge Prof. Jean-Luc Veuthey (School of Pharmaceutical Sciences, University of Geneva, Switzerland) for scientific discussions, suggestions and corrections. Davy Guillarme also wishes to thank the Swiss National Science Foundation for support through a fellowship to Szabolcs Fekete (31003A_159494).

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Figure captions

Figure 1: Main properties of different HPLC vial inserts types used in this study.

Figure 2: Relative change of peak areas observed during four consecutive injections of mAb parent samples.

Figure 3: Relationship between injection repeatability and mAb parent formulation concentration. Relative change of peak areas between the first and second injections was plotted vs. the mAb concentration.

Figure 4: Relative decrease of peak areas (adalimumab) in the third injection relative to the first injection for six different types of inserts and at different time points (day 1, day 4, day 10 and day 15). Panels A and B present two different series of experiments differing in the homogenization procedure. The yellow areas indicate the experiments where samples were homogenized. Letters A, B, C, D, E and F stand for the vial types, as described in figure 1.

Figure 5: Comparison of consecutive peak areas observed with a reference vial (without insert) and with the A type insert obtained in the two series of experiments at different days. Absolute peak areas were plotted as a function of time that adalimumab spent in vial insert.

Figure 6: Change of peak area during five consecutive injections of native adalimumab (panel A) and its dimer (panel B) after 50 minutes residence time prior to injection. Sample was injected from C type vial insert.

Figure 7: Representative SEC chromatograms of five consecutive injections of adalimumab (conditions as described in figure 6).

533 Figure 8: Decrease of observed peak areas when injecting with a needle offset position at 2
534 mm and 10 mm from the bottom. Adalimumab was injected from C type vial insert.

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536 Figure 9: Schematic view of possible processes and parameters that can affect the
537 repeatability of chromatographic data when analyzing mAbs.

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code	A		B		C		D		E		F
volume (μL)	150		200		300		250		250		300
size (mm)	31 x 5		31 x 5		31 x 6		31 x 6		29 x 5.7		32 x 11.6
tip (mm)	15		9		12		15		n.a.		-
diameter	narrow		narrow		large		large		narrow		large
material	glass		glass		glass		glass-silanized		glass-silanized		glass
shape	conical (narrow at the bottom)		conical (normal at the bottom)		conical (narrow at the bottom)		conical (normal at the bottom)		conical (normal at the bottom)		conical (triangular shape)
comment											

539 Figure 1.

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542 Figure 2.

Figure 3.

Figure 4.

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Figure 6.

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Figure 7.

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575 Figure 9.
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