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Research paper

Association of ranibizumab (Lucentis®) or bevacizumab (Avastin®) with dexamethasone and triamcinolone acetonide: An *in vitro* stability assessment

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ABSTRACT

The *in vitro* stability of monoclonal antibodies used for age-related macular degeneration, ranibizumab and bevacizumab, was investigated. The aggregation profile of the antibodies was compared, alone and after association with dexamethasone sodium phosphate or triamcinolone acetonide. Commercial formulations of ranibizumab and bevacizumab were dialysed into three different buffers. After dialysis, samples were stored at 4 °C, 25 °C and 40 °C during 35 days, alone and in combination with dexamethasone sodium phosphate, triamcinolone acetonide phosphate solution or triamcinolone acetonide suspension. Combined formulations based on both commercial formulations were investigated as well. The aggregation state of the antibodies was measured by multi-angle light scattering (MALS) after separation by asymmetrical flow field-flow fractionation (AFFF) or size-exclusion chromatography (SEC). Ranibizumab results to be more stable than bevacizumab, alone and in combination with dexamethasone sodium phosphate or triamcinolone acetonide. Elevation in concentration, pH and temperature causes a decrease in stability of both antibodies. The association of triamcinolone acetonide phosphate solution with either ranibizumab or bevacizumab is observed to be the least stable combination of all samples tested. Dexamethasone sodium phosphate was shown to have a stabilizing effect on bevacizumab, although this is not the case for its combination with the commercial formulation Avastin®. The results demonstrate that the *in vitro* association of either ranibizumab or bevacizumab with dexamethasone sodium phosphate or triamcinolone acetonide suspension does not decrease the stability of these antibodies. Although ranibizumab is more stable than bevacizumab *in vitro*, further research has to point out how this affects their mechanism of action in vivo.

1. Introduction

Ranibizumab (Lucentis®) is a humanized monoclonal antibody fragment with a molecular weight of 48 kiloDalton (kDa) that inhibits vascular endothelial growth factor (VEGF). It has been registered since 2006 for the treatment of neovascular age-related macular degeneration (AMD) [1,2]. Bevacizumab (Avastin®) is a monoclonal humanized antibody with a molecular weight of 149 kDa that has a comparable mechanism of action, since ranibizumab is a fragment of the same antibody and currently, is widely used off-label for the treatment of AMD [1]. For ranibizumab, a monthly injection is recommended to maintain therapeutically effective drug concentrations [3], and the same frequency is generally reported for bevacizumab injections [2]. Nevertheless, a reduced frequency of injections would be favourable because of patient discomfort and risk of complications [4].

Combination therapy of VEGF-inhibitors with anti-inflammatory drugs such as dexamethasone or triamcinolone acetonide could possibly increase the therapeutic efficacy of the treatment. Anti-inflammatory drugs are well known for their positive effects on AMD [5–8], with a different mechanism of action compared to VEGF-inhibitors [9]. Therefore, combination of the two drugs could lead to a synergistic effect.

Several clinical trials report the co-administration of bevacizumab with triamcinolone acetonide or dexamethasone [5,10–13]. However, in these studies, the possible interaction between bevacizumab and triamcinolone acetonide or dexamethasone has never been taken into account. The antibody-based formulation may aggregate, resulting in serious clinical side-effects, since protein aggregates can reduce the efficacy and enhance the immunogenicity of the protein drug [14–17]. Thus, formation of dimers, trimers or higher order oligomers should be prevented where possible.

The present study focuses on the *in vitro* compatibility of ranibizumab and bevacizumab with dexamethasone sodium

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phosphate, triamcinolone acetonide phosphate solution and triamcinolone acetonide suspension. The purpose is to investigate whether the monomeric native state of the antibodies is influenced by addition of these anti-inflammatory drugs.

Samples are analysed by asymmetrical flow field-flow fractionation (AFFF). This analytical technique covers a large range of detectable protein sizes, from protein monomers up to subvisible particles [18], which makes it a good candidate for protein aggregation studies. As described by Demeule, AFFF offers the possibility to combine two methodologies [19]: in a first step, hardly any mechanical stress is applied allowing the detection of loose aggregates, followed by a second step in which the different fractions of monomers and aggregates are separated.

2. Methods

Four different series of samples were tested.

2.1. Series I

To obtain a baseline, the aggregation state of both commercial formulations of ranibizumab (Lucentis®, Novartis Pharma Schweiz AG, Bern, Switzerland) and bevacizumab (Avastin®, Roche Pharma, Reinach, Switzerland) was analysed during 35 days of storage at 4 °C, 25 °C and 40 °C. For both formulations, three different sample containers were stored, and for each container, two analyses were carried out directly after preparation (t_0) and after 7, 14 and 35 days. Because of the low inter-sample variability over time shown in these analyses (CV $\leqslant$ 0.5% for both ranibizumab and bevacizumab at all time points and temperatures), all other analyses were carried out in duplicate.

2.2. Series II

Concentrations of 5, 10, 18 and 25 mg/ml ranibizumab and bevacizumab were prepared to study the influence of concentration on the aggregation state. Ranibizumab and bevacizumab were dialysed overnight (Pierce Slide-A-Lyzer Dialysis Cassette, Reactolab, Servion, Switzerland) into a 10 mM histidine buffer pH 5.5 and a 50 mM phosphate buffer pH 6.2, respectively, since these are the buffers used in the commercial products Lucentis® and Avastin®. NaCl was added to the buffers to obtain isotonicity. After dialysis, all samples were concentrated by centrifugation or diluted with buffer to concentrations of 5, 10, 18 and 25 mg/ml. Samples were stored during 35 days at 40 °C.

2.3. Series III

The commercial products Lucentis® and Avastin® were associated with dexamethasone 21-phosphate disodium salt (Sigma–Aldrich, Lausanne, Switzerland), triamcinolone acetonide-21-phosphate dipotassium salt solution (Kenacort A solubile, Dermapharm AG Arzneimittel, Grünwald, Germany) or triamcinolone acetonide suspension (Kenacort A 40, Dermapharm AG Arzneimittel, Grünwald, Germany) to study the influence of a combined formulation on the stability of the antibody.

Both antibodies were stored at 40 °C, alone and in combination with dexamethasone sodium phosphate, triamcinolone acetonide solution and triamcinolone acetonide suspension. Based on concentrations described in literature for combination therapy with bevacizumab, the following combined formulations were selected:

i. 1.5 mg bevacizumab and 0.8 mg dexamethasone sodium phosphate [5]
ii. 1.25 mg bevacizumab and 2 mg triamcinolone acetonide [10–13] (solution and suspension)
iii. 0.6 mg ranibizumab and 0.8 mg dexamethasone sodium phosphate
iv. 0.5 mg ranibizumab and 2 mg triamcinolone acetonide (solution and suspension)

The dosage of ranibizumab in the combined formulation was chosen to be 2.5 times lower than that of bevacizumab, based on the difference in therapeutic dosage, which is 0.5 mg for ranibizumab compared to 1.25 mg for bevacizumab. The pH of the combined formulations was not adjusted after addition of the anti-inflammatory drugs, in order to stay as close as possible to the clinical studies mentioned earlier.

2.4. Series IV

To evaluate the influence of anti-inflammatory drugs on the antibodies in different buffers and at different pH values, bevacizumab and ranibizumab were associated with dexamethasone 21-phosphate disodium salt, triamcinolone acetonide-21-phosphate dipotassium salt solution or triamcinolone acetonide suspension. Before addition of the anti-inflammatory drugs, both bevacizumab and ranibizumab were dialysed overnight in three different isotonic buffers to change the pH. For bevacizumab, 50 mM acetate buffer pH 5.0, 50 mM phosphate buffer pH 6.2 and 50 mM phosphate buffer pH 7.0 were used. For ranibizumab, 50 mM acetate buffer pH 5.0, 10 mM histidine buffer pH 5.5 and 50 mM phosphate buffer pH 7.0 were chosen. The buffer choice was based on a pH range and buffer capacity that is tolerated by the eye and that is acceptable for the stability of the antibodies [20,21]. A phosphate buffer pH 6.2 and histidine buffer pH 5.5 were selected because these buffers are used in the commercial products Avastin® and Lucentis®, respectively.

After dialysis, bevacizumab was analysed at a concentration of 19 mg/ml (pH 5.0 = 19.8 mg/ml, pH 6.2 = 19.2 mg/ml, pH 7.0 = 18.7 mg/ml) and ranibizumab at 6 mg/ml (pH 5.0 = 6.8 mg/ml, pH 5.5 = 5.9 mg/ml, pH 7.0 = 6.4 mg/ml). Both antibodies were stored at 4 °C, 25 °C and 40 °C, alone and in combination with dexamethasone sodium phosphate, triamcinolone acetonide solution and triamcinolone acetonide suspension. The ratio antibody : anti-inflammatory drug was the same as described in Series III. Again, no pH adjustments were made after addition of the anti-inflammatory drug, to mimic the clinical studies in which the combinations were applied.

2.5. Sample analysis

Samples with a sample volume of 0.5 μl for all bevacizumab samples and 1.0 μl for all ranibizumab samples were analysed directly after preparation (t_0) and after 7, 14 and 35 days. Before analysis, the samples containing triamcinolone acetonide suspension were filtered over a 0.2 μm filter to obtain a visible clear sample for measurement. The weight-averaged molar mass of the antibody fractions was measured by multi-angle light scattering (MALS) after separation by asymmetrical flow field-flow fractionation (AFFF) (Wyatt Technology Europe GmbH, Dernbach, Germany) [22–24]. Since the molar masses of both Avastin and Lucentis are known, the data obtained by MALS can be used to calculate the degree of aggregation. The concentrations of bevacizumab and ranibizumab were determined by UV spectroscopy at 280 nm, based upon an extinction coefficient of 1.7 and 1.8 cm ml/mg, respectively. Data were collected and analysed with Astra software (Wyatt Technology Europe GmbH, Dernbach, Germany). The aggregation state was expressed as the percentage of monomers versus time. For the aggregated fraction, a distinction

was made between dimers, aggregates that are greater than or equal to trimers and higher order aggregates ($\geqslant$10 times $MW_{monomer}$). The mobile phase was the same as the buffer used for each analysed formulation. To minimize mechanical stress applied on the sample during analysis by focussing and cross-flow, samples were analysed without focussing and cross-flow in a first step. In a second step, focussing and cross-flow were applied in order to separate the fraction of monomers from any existing aggregates.

The samples containing the commercial products Avastin® and Lucentis® in combination with anti-inflammatory drugs (Series III) were analysed by Size-Exclusion Chromatography as well, in order to have an orthogonal technique to double check the results obtained by AFFF. Both analyses (AFFF and SEC) were performed on the same samples. Separations were achieved on an Ultrahydrogel 120 (Lucentis® samples) and an Ultrahydrogel 250 column (Avastin® samples) (Waters, Milford, USA). The weight-averaged molar mass and concentrations of both antibodies were measured by MALS and UV spectroscopy, respectively. Analyses were carried out in isocratic conditions and at a temperature of 35 °C, using a 50 mM phosphate buffer as mobile phase.

3. Results

3.1. Series I

The commercial product Lucentis® is very stable at 4 °C, 25 °C and 40 °C (Fig. 1A). As expected, a rise in temperature causes a small decrease in stability: even at 40 °C, an average monomer percentage of 99.6 ± 0.3% (n = 6) is observed after 35 days of storage. However, the small fractions of aggregates present are higher order aggregates.

The commercial product Avastin® is less stable than Lucentis® (Fig. 1B). At t = 0, the average measured monomer percentage is 97.2 ± 0.1% (n = 6). After 35 days of storage, this percentage decreases to 96.9 ± 0.2% (n = 6) at 4 °C, 96.6 ± 0.1% (n = 6) at 25 °C and 91.3 ± 0.5% (n = 6) at 40 °C. The measured aggregates are present in the form of dimers.

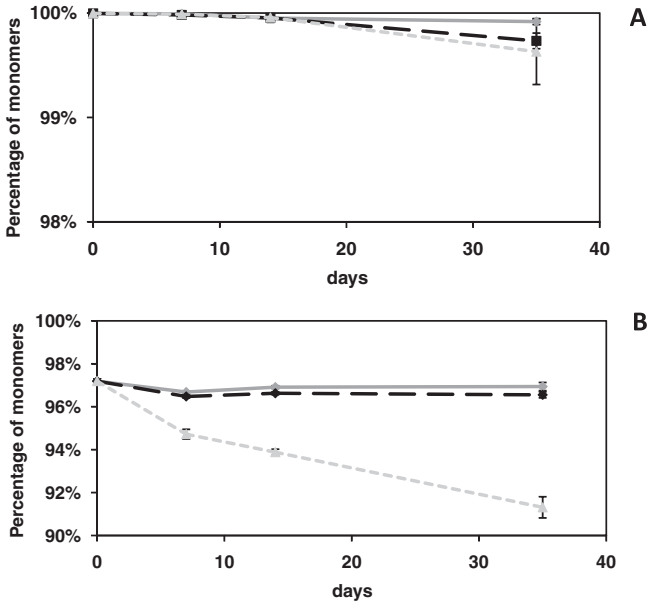

Fig. 1. Comparison of commercial products of ranibizumab and bevacizumab at different storage temperatures. (A) Upper figure represents the percentage of monomers of the commercial product of ranibizumab (Lucentis®) after storage at 4 °C (n = 6, dark grey line), 25 °C (n = 6, dashed black line) and 40 °C (n = 6, dashed light grey line). (B) Lower figure represents the percentage of monomers of the commercial product of bevacizumab (Avastin®) after storage at 4 °C (n = 6, dark grey line), 25 °C (n = 6, dashed black line) and 40 °C (n = 6, dashed light grey line).

3.2. Series II

The comparison of ranibizumab and bevacizumab at 5, 10, 18 and 25 mg/ml also shows a higher stability for ranibizumab than for bevacizumab at all four concentrations (Fig. 2A and B). A rise in concentration causes a stability decrease for both antibodies. After 35 days of storage at 40 °C, the average percentage of monomers is 99.8% (n = 2) for a concentration of 5 mg/ml ranibizumab. For concentrations of 10, 18 and 25 mg/ml ranibizumab, this percentage rises to 98.2% (n = 2), 97.0% (n = 2) and 97.5% (n = 2), respectively. For all concentrations, the aggregated fraction consists of dimers.

98.1% (n = 2) of bevacizumab is found to be present as monomer at a concentration of 5 mg/ml after 35 days at 40 °C, compared to 97.2% (n = 2) at 10 mg/ml, 92.9% (n = 2) at 18 mg/ml and 92.8% (n = 2) at 25 mg/ml. The concentration of 5 mg/ml demonstrates to contain only dimers in the aggregated fraction, the other concentrations contain dimers and a fraction $\geqslant$trimers. After 35 days, a partial reversion to monomers is detected for the samples with concentrations of 5 mg/ml and 10 mg/ml (Fig. 2B). Almost no difference is observed between the samples with a concentration of 18 mg/ml and 25 mg/ml.

3.3. Series III

3.3.1. Lucentis® commercial product, alone and in combination with dexamethasone sodium phosphate, triamcinolone acetonide phosphate solution and triamcinolone acetonide suspension

The commercial product Lucentis® stays stable over time after storage at 40 °C, alone and in combination with dexamethasone and triamcinolone acetonide suspension (Table 1A). After 35 days,

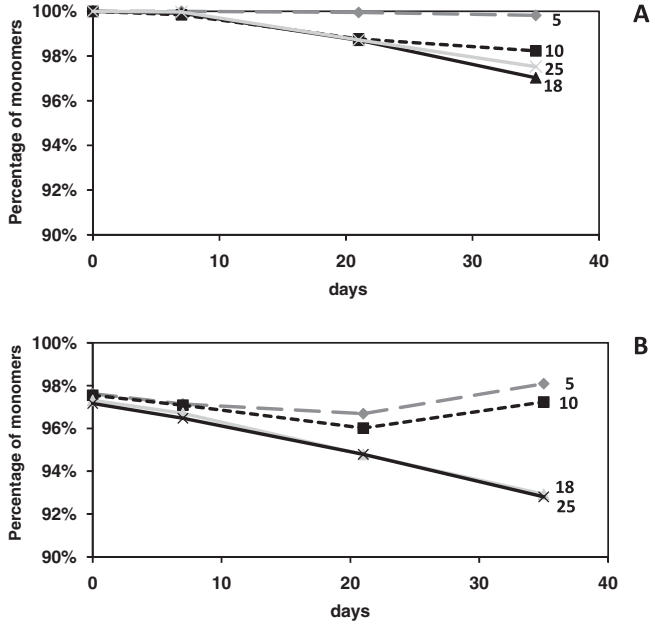

Fig. 2. Influence of concentration on the stability of ranibizumab and bevacizumab. The stability of both antibodies is tested at four different concentrations and stored at 40 °C. (A) Upper figure shows the average percentage of monomers (n = 2) of ranibizumab 5 mg/ml (grey dashed line), ranibizumab 10 mg/ml (black dashed line), ranibizumab 18 mg/ml (dark grey line) and ranibizumab 25 mg/ml (light grey line), pH 5.5. (B) Lower figure shows the average percentage of monomers (n = 2) of bevacizumab 5 mg/ml (grey dashed line), bevacizumab 10 mg/ml (black dashed line), bevacizumab 18 mg/ml (light grey line) and bevacizumab 25 mg/ml (dark grey line), pH 6.2.

Table 1
Comparison of the aggregation state of Lucentis® alone and as combined formulation. (A) Data obtained by AFFF, expressed as the percentage of monomers and aggregates ± SD. (B) Data obtained by SEC, expressed as the percentage of monomers and aggregates ± SD. Samples are stored for 35 days at 40 °C ($n = 3$).

	Lucentis (%)	Lucentis + dexamethasone (%)	Lucentis + triamcinolone suspension (%)	Lucentis + triamcinolone solution
(A) Results obtained by AFFF separation				
Monomer	99.5 ± 0.1	99.3 ± 0.1	98.7 ± 0.1	Large visible aggregates
Dimer				
⩾Trimer	0.5 ± 0.1	0.7 ± 0.1		
Higher order aggregates			0.7 ± 0.1	
(B) Results obtained by SEC separation				
Monomer	99.7 ± 0.2	98.9 ± 0.2	99.35 ± 0.2	Large visible aggregates
Dimer				
⩾Trimer	0.3 ± 0.2	1.1 ± 0.2		
Higher order aggregates			1.1 ± 0.2	

Table 2
Comparison of the aggregation state of Avastin® alone and as combined formulation. (A) Data obtained by AFFF, expressed as the percentage of monomers and aggregates ± SD. (B) Data obtained by SEC, expressed as the percentage of monomers and aggregates ± SD. Samples are stored for 35 days at 40 °C ($n = 3$).

	Avastin (%)	Avastin + dexamethasone (%)	Avastin + triamcinolone suspension (%)	Avastin + triamcinolone solution
(A) Results obtained by AFFF separation				
Monomer	88.5 ± 0.4	88.6 ± 0.9	89.6 ± 3.6	Large visible aggregates
Dimer	11.5 ± 0.4	10.2 ± 0.8	10.4 ± 3.6	
⩾Trimer				
Higher order aggregates		1.2 ± 0.3		
(B) Results obtained by SEC separation				
Monomer	95.0 ± 0.3	88.3 ± 0.4	96.1 ± 0.1	Large visible aggregates
Dimer	5.0 ± 0.3		3.9 ± 0.1	
⩾Trimer				
Higher order aggregates		11.7 ± 0.4		

monomer percentages of 99.5%, 99.3% and 98.7% are observed by AFFF analysis for Lucentis® alone, in combination with dexamethasone and with triamcinolone acetonide suspension, respectively. For all three formulations, the aggregated fraction is present in the form of higher order aggregates. These results are confirmed by SEC analysis (Table 1B). The combined formulation of Lucentis® with triamcinolone acetonide solution is unstable; large visible aggregates were observed after 7 days storage. Therefore, this sample was not further analysed.

3.3.2. Avastin® commercial product, alone and in combination with dexamethasone sodium phosphate, triamcinolone acetonide phosphate solution and triamcinolone acetonide suspension

The combined formulation of Avastin® and triamcinolone acetonide phosphate solution shows large visible aggregates as well after 14 days of storage at 40 °C. The other combinations demonstrate a similar amount of aggregation as the Avastin® alone: after 35 days, monomer percentages of 88.5%, 88.6% and 89.6% are detected for Avastin® alone, in combination with dexamethasone phosphate and with triamcinolone acetonide suspension, respectively. However, the aggregated fraction consists of only dimers for Avastin® alone and for the formulation containing triamcinolone acetonide suspension, whereas the combination with dexamethasone phosphate shows a fraction of dimers and a small fraction of higher order oligomers as well (Table 2A). The results are confirmed by SEC. The percentages of monomers for Avastin® and the combination with triamcinolone acetonide suspension are slightly lower than obtained by AFFF; however, the aggregated fraction also contains dimers only. For the formulation with dexamethasone phosphate, a similar monomer percentage is found as by AFFF; however, the fraction of aggregates consists only of higher order aggregates, probably because the dimer peak and the higher order oligomer peak are not separated (Table 2B).

3.4. Series IV

3.4.1. Ranibizumab, alone and in combination with dexamethasone sodium phosphate, triamcinolone acetonide phosphate solution and triamcinolone acetonide suspension

Ranibizumab is observed to be very stable at all pH conditions (Table 3A). The sample of ranibizumab alone is least stable at pH 7, with an average monomer percentage of 98.7% ($n = 2$) after 35 days of storage at 40 °C (Fig. 3). On the other hand, at both pH 5.0 and pH 5.5, the sample shows a 100% monomer state during 14 days at all temperatures (data not shown). At 35 days, a monomer percentage >99% is observed for these samples. For all three pHs, the aggregated fraction contains oligomers ⩾ trimers.

Ranibizumab in association with dexamethasone sodium phosphate presents a similar degree of aggregation as ranibizumab alone under all three pH conditions tested at 4 °C and 25 °C. At 40 °C, the addition of dexamethasone causes a slightly higher aggregation at pH 5.0 and 5.5, i.e. from >99% of monomers to >95%. The measured aggregates are present as dimers. At pH 7.0, the aggregation profile of the combined sample at 40 °C is comparable to that of ranibizumab alone; both show monomer percentages >98% and a fraction ⩾trimers after 35 days of storage at 40 °C.

The combination of ranibizumab with triamcinolone acetonide phosphate solution causes an increase in aggregation at all pH values compared to ranibizumab alone. The sample stored at pH 7, 40 °C presents the highest amount of aggregates (⩾trimers), with an average monomer percentage of 93.9% ($n = 2$). At pH 5.0 and pH 5.5, monomer percentages of 96.7% ($n = 2$, pH 5.0) and 98.7% ($n = 2$, pH 5.5) are obtained after 35 days of storage at 40 °C. Oligomers ⩾ trimers are detected as aggregated fraction. Ranibizumab combined with triamcinolone acetonide suspension also shows a higher aggregation at pH 7.0 compared to ranibizumab alone, with an average monomer percentage of 95.9% ($n = 2$). At pH 5.0 and 5.5,

Table 3
Comparison of the aggregation state of ranibizumab (A) or bevacizumab (B) alone and as combined formulation. Results are expressed as the average percentage of monomers and aggregates ($n = 2$). Samples are stored for 35 days at 40 °C.

	Ranibizumab			Ranibizumab + dexamethasone			Ranibizumab + triamcinolone suspension			Ranibizumab + triamcinolone solution		
(A) Percentages of monomers and aggregates of ranibizumab												
pH	**5**	**5.5**	**7**	**5**	**5.5**	**7**	**5**	**5.5**	**7**	**5**	**5.5**	**7**
Monomer	99.2%	99.5%	98.7%	95.5%	95.3%	98.8%	99.3%	99.4%	95.9%	96.7%	98.7%	93.9%
Dimer				4.5%	4.7%							
⩾Trimer	0.8%	0.5%	1.3%			1.2%	0.7%	0.6%	4.1%	3.3%	1.3%	6.1%
	Bevacizumab			Bevacizumab + dexamethasone			Bevacizumab + triamcinolone suspension			Bevacizumab + triamcinolone solution		
(B) Percentages of monomers and aggregates of bevacizumab												
pH	**5**	**6.2**	**7**	**5**	**6.2**	**7**	**5**	**6.2**	**7**	**5**	**6.2**	**7**
Monomer	95.9%	87.5%	68.8%	94.4%	95.2%	95.6%	Large visible aggregates	84.2%	73.3%	96.5%	Large visible aggregates	60.6%
Dimer	4.1%	12.5%	27.2%	5.6%	4.8%	4.4%		15.8%	26.7%	3.5%		2.2%
⩾Trimer			4.0%									16.4%
Higher order aggregates												20.8%

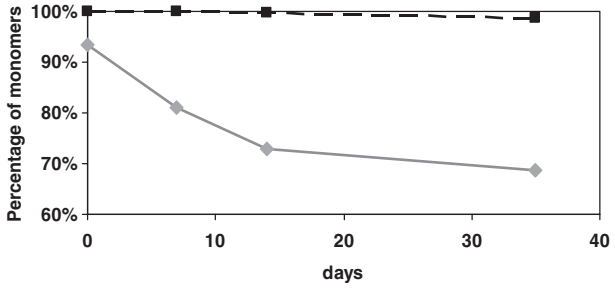

Fig. 3. Stability comparison of ranibizumab (dashed black line) and bevacizumab (grey line) at pH 7.0, 40 °C. Analyses are carried out in duplicate; the average percentage of monomers of the two analyses is taken for both curves.

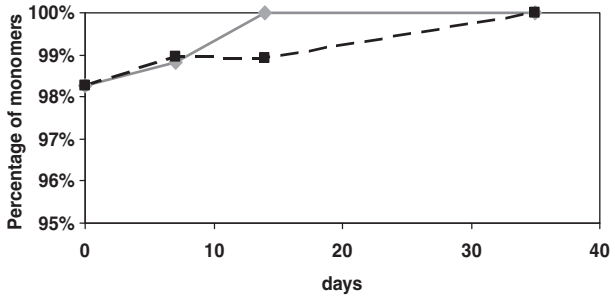

Fig. 4. Reversion back into monomers of bevacizumab in pH 5.0, after storage at 4 °C (grey line) and 25 °C (dashed black line). Analyses are carried out in duplicate; the average percentage of monomers of the two analyses is taken for both curves.

the aggregation state is similar to that of the ranibizumab sample. For all three pHs, the aggregated fraction is ⩾trimers.

3.4.2. Bevacizumab, alone and in combination with dexamethasone sodium phosphate, triamcinolone acetonide phosphate solution and triamcinolone acetonide suspension

After changing the pH from 6.2 to 5.0 by dialysis, the monomer percentage of the sample of bevacizumab alone rises from 98.3% (t_0, $n = 2$) to 100% ($n = 2$) after storage at 4 °C and 25 °C (Fig. 4). This stabilization is not observed after 35 days of storage at 40 °C: an average monomer percentage of 95.9% ($n = 2$) and 4.1% of dimers is measured. Storage at pH 6.2 and pH 7.0 results in monomer percentages of 87.5% ($n = 2$) and 68.8% ($n = 2$), respectively (Table 3B).

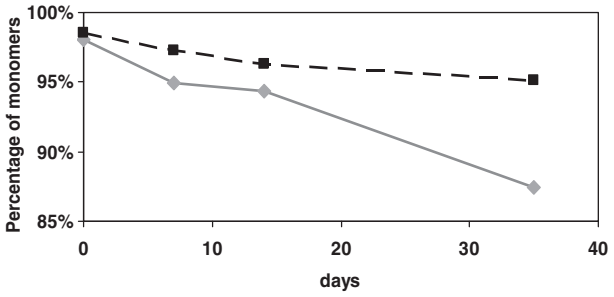

Fig. 5. Stabilizing effect of dexamethasone sodium phosphate on bevacizumab at pH 6.2, 40 °C. The monomer percentage of the sample of bevacizumab alone is represented by the grey line and the combination sample of dexamethasone sodium phosphate and bevacizumab by the dashed black line. Analyses are carried out in duplicate; the average of the two analyses is taken for both curves.

The aggregated fraction demonstrates only dimers for pH 6.2, whereas pH 7.0 contains dimers and a fraction of oligomers ⩾ trimers.

The association of bevacizumab with dexamethasone sodium phosphate causes a surprising stabilization of the antibody in comparison with the sample of bevacizumab alone (Fig. 5). After 35 days of storage at 40 °C at pH 6.2 and pH 7.0, the combination sample shows average monomer percentages of 95.2% ($n = 2$) for pH 6.2 and 95.6% ($n = 2$) for pH 7.0. The effect is not observed at pH 5, after 35 days at 40 °C, a monomer percentage of 94.4% ($n = 2$) is measured. For all three pH conditions, the aggregated fraction consists of dimers.

The combination of bevacizumab with triamcinolone acetonide phosphate solution is the least stable of all samples tested, with an average monomer percentage of 60.6% ($n = 2$) at pH 7.0 after 35 days of storage at 40 °C. The aggregated fraction contains oligomers ⩾ trimers and higher order aggregates. At pH 6.2, large visible aggregates are observed after 35 days of storage at 40 °C. After filtration over a 0.2 μm filter, neither monomers nor aggregates are detected during AFFF analysis of the sample. Therefore, this sample is left out of the results. The most stable combination with triamcinolone acetonide phosphate solution is observed at pH 5.0, with an average monomer percentage of 96.5% ($n = 2$) and 3.5% dimers after 35 days storage at 40 °C. The association of bevacizumab with triamcinolone acetonide suspension forms a visible precipitation at pH 5.0. The combination is filtered over a 0.2 μm filter prior to analysis. Analysis of the filtrate shows no detectable amount of monomers or aggregates and is therefore left out of the results.

At pH 6.2 and pH 7.0, the amount of aggregation is similar to the one of bevacizumab alone, and the aggregated fractions are both observed to be present as dimers.

4. Discussion

The purpose of this study is to compare the stability of ranibizumab and bevacizumab alone and in combination with anti-inflammatory drugs. Percentages of monomers, dimers, trimers and higher order aggregates of both antibodies are measured to determine whether association with dexamethasone sodium phosphate and triamcinolone acetonide influences the aggregation state of these proteins. Since aggregates have been observed to cause severe side-effects [14–17], these results are of great importance for clinical use.

A difference in stability is already observed between both commercial products; for Lucentis®, an average monomer percentage of 99.6 ± 0.3% ($n = 6$) is measured after 35 days of storage at 40 °C, compared to 91.3 ± 0.5% ($n = 6$) for Avastin®. The stability difference might be explained by the fact that the concentration of ranibizumab is approximately 2.5 times lower than that of bevacizumab: aggregation processes can be concentration dependent [21,25]; and therefore, a more concentrated formulation may result in a higher state of aggregation. To exclude the possibility that ranibizumab is more stable because of its lower concentration in comparison with bevacizumab, the aggregation state of both antibody samples is compared at concentrations of 5, 10, 18 and 25 mg/ml. As expected, both antibodies show a decrease in stability after increasing the concentration. However, a comparison of ranibizumab and bevacizumab at the same concentration shows a higher stability for ranibizumab at all four concentrations (Fig. 2A and B). Thus, the fact that bevacizumab is 2.5 times more concentrated than ranibizumab cannot totally explain the difference in stability.

The pH variation between both commercial formulations (pH 5.5 for Lucentis versus pH 6.2 for Avastin) might also be a factor contributing to the observed difference in stability. At pH 5.0, bevacizumab is very stable as well; for both bevacizumab alone and associated with dexamethasone sodium phosphate, a reversion back into monomers is observed with time. Apparently, pH 5.0 is an optimal environment for bevacizumab; aggregates that are formed at pH 6.2 in the commercial product revert back into monomers at a lower pH. A similar reversion back into monomers is detected with the samples of 5 mg/ml and 10 mg/ml bevacizumab after 35 days of storage. The results are confirmed by a study of Moore et al. [21], who observed that the ideal pH for the native protein structure of bevacizumab is pH 5.5 and below. Furthermore, this work showed the capacity of bevacizumab to revert back into monomers upon dilution.

Based on the stability results of the present study, one could state that ranibizumab is a therapeutically better option compared to bevacizumab. However, since protein characteristics are very specific, it is unknown what the effect of the aggregation profiles of bevacizumab and ranibizumab will be *in vivo*. At this stage of investigation, it is uncertain whether bevacizumab with its higher aggregation state will have a higher clinical significance compared to the more stable ranibizumab. Besides, it must be taken into account that although the aggregated fraction for ranibizumab is very small, the aggregates observed are higher order aggregates, compared to dimers for bevacizumab. Since the last has the unique ability to revert back to the monomeric state upon dilution [21], the formation of dimers might have advantages for the intravitreal usage of bevacizumab. As the formed dimers are probably too big to cross the retina, it might be possible that they will revert back into monomers in the vitreous, thereby causing a prolonged release effect *in vivo*. Thus, although ranibizumab is more stable than bevacizumab *in vitro*, it is uncertain how this affects their mechanisms of action *in vivo*. Further research will focus on the effects of the aggregation state of both antibodies *in vivo*.

The percentage of aggregation of bevacizumab alone and in the combination samples rises with higher pH and temperature. This can be explained by a low range in thermodynamic stability of the native protein structure [26]. Changes in external factors like pH or temperature can easily cause a destabilization in the structure of the protein, which leads to unfolding of parts of the protein. Partly unfolded proteins are more prone to aggregation than the native state of the protein [26]. For ranibizumab, similar effects are observed with an augmentation in temperature and less clear, for a rise in pH. All samples of ranibizumab alone and in combination that are stored at pH 5.0 and pH 5.5 show comparable results, and their pHs probably lie too close together to show an effect. At pH 7.0, a rise in aggregation percentage is observed for all samples, except for the sample containing ranibizumab and dexamethasone. However, changes are minimal since all ranibizumab samples stay very stable.

For the combination samples, the most surprising effect observed is the stabilization of bevacizumab after addition of dexamethasone sodium phosphate. The influence of dexamethasone sodium phosphate was only observed at values of pH 6.2 and pH 7.0; at pH 5.0, bevacizumab is already very stable on its own. For the commercial formulation, a similar effect was observed after 7 and 14 days storage at 40 °C. However, after 35 days, a fraction of large aggregates was detected for this combined formulation. The presence of large aggregates in the commercial formulation and the absence of these aggregates in the dialysed formulations show that the environment can highly influence the conformation of the antibody. Apparently, the ability of dexamethasone sodium phosphate to partly inhibit aggregation of the antibody only exists under certain circumstances. Consequently, optimisation of the formulation before starting clinical studies is a crucial step in combining therapeutic antibodies. It is unlikely that the stabilizing effect is caused by a pH change, since the pH was elevated after addition of dexamethasone sodium phosphate: a pH of 6.6 was measured for the sample of pH 6.2 and a pH 7.2 for the sample of pH 7.0. Since a higher pH leads to more aggregation, the stabilization is probably not due to changes in the pH.

The association of both antibodies with triamcinolone acetonide suspension is more stable than the combination with the triamcinolone acetonide phosphate solution. This might be caused by the fact that the pH of the triamcinolone acetonide phosphate solution alone is 7.4, whereas that of the suspension is 6.2. Therefore, the addition of triamcinolone acetonide phosphate solution causes a higher pH raise for all combinations than that of triamcinolone acetonide suspension, resulting in a higher aggregation state. Besides, where all other combined formulations are isotonic, a highly hypertonic formulation is formed after addition of triamcinolone acetonide solution to the antibody, and this environmental change might also result in a destabilized antibody.

Dexamethasone sodium phosphate was added as a solid form that was solved in the bevacizumab or ranibizumab formulation. Therefore, the association did not change the concentration of the antibodies. For triamcinolone acetonide, a solution and a suspension were used, thereby diluting the concentration of bevacizumab and ranibizumab. This may have influenced the stability of the antibodies, since less concentrated formulations are generally less prone to aggregation.

5. Conclusions

In this study, an *in vitro* comparison of the aggregation state of bevacizumab and ranibizumab was made, and the effects of dexamethasone sodium phosphate and triamcinolone acetonide

on their stability were investigated. The purpose of the study was not to determine whether bevacizumab or ranibizumab is a therapeutically better choice, but to provide a preliminary insight in the possible interactions between these antibodies and anti-inflammatory drugs. Ranibizumab is observed to be more stable than bevacizumab alone and in combination with dexamethasone sodium phosphate and triamcinolone acetonide suspension. Nevertheless, it is unknown how the *in vivo* activity of both antibodies is affected by the aggregates that are formed. Work is in progress to test the effects of the aggregation state of these antibodies *in vivo*. The results show that the stability profile of ranibizumab and bevacizumab is generally not decreased by the addition of dexamethasone sodium phosphate or triamcinolone acetonide suspension. A stabilizing effect of dexamethasone sodium phosphate on bevacizumab is even observed. However, combination of the commercial product Avastin® with dexamethasone phosphate shows higher order aggregates after 35 days storage at 40 °C, demonstrating the importance of optimising a combined formulation before starting clinical studies. Further research has to determine whether a combined formulation is able to prolong the injection interval.

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