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Separation and quantification of a novel two-component vaccine adjuvant

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Abstract

Two reversed-phase HPLC methods were developed for the quantitative determination of the two components of the novel vaccine adjuvant IC31. The adjuvant consists of a mixture of a synthetic oligodeoxynucleotide (ODN) and an 11-mer cationic peptide. The negatively charged oligodeoxynucleotide and the positively charged peptide form a complex that has to be quantitatively dissociated for analysis. Dissociation of the complex was achieved with a basic heparin solution (1000 IU/ml) when analyzing the ODN, whereas 30% acetic acid was used for the determination of the peptide. Both methods are suitable for identification and quantification but also for stability indicating investigations.

Keywords: Cationic peptide; Complex dissociation; Heparin; ODN; Reversed-phase HPLC

1. Introduction

Traditional vaccines consist of live attenuated pathogens, whole inactivated organisms, purified components thereof or inactivated toxins [1]. Despite their success in the past, alternatives have to be developed for several reasons, including undesirable adverse events caused by components contained in traditional vaccines. Furthermore, adjuvants used in approved vaccines predominantly induce B cell responses, but not T cells. Clearly, the trend is away from complex vaccines containing complete organisms towards well-defined products that only contain highly pure components and only few essential antigens [1,2]. Unfortunately, defined, highly pure antigens are also known to be less immunogenic than traditional vaccines [2,3]. Hence, novel adjuvants are required to enhance their immunogenicity, especially to induce cell-mediated immune responses [1,4,5].

The best-known adjuvants, which also have extensive track records of safety and adjuvanticity with a number of antigens, are aluminum compounds [6,7]. Although widely used, their application is often limited due to side effects, lack of inducing potent immune responses with some antigens but also due to the inability to augment cell-mediated immune responses, especially cytotoxic T-cell responses.

Therefore, we are developing adjuvants, which predominantly induce Th-1 type responses. As shown previously, the synthetic polymer poly-L-arginine [8] in combination with synthetic oligodeoxynucleotides containing CpG-motifs turned out to fulfill this requirement [9]. Recently, we have developed a new variety of this type of adjuvant, named IC31. In this case, an 11-mer cationic peptide (KLK) [10] and a synthetic oligodeoxynucleotide (ODN1a) are combined to increase the immune response to a variety of antigens like peptides or proteins. A major aspect, beside the pharmacological/toxicological mode of action and efficacy of an adjuvant, is the ability to monitor production and to release

Abbreviations: AUC, area under the curve; HFIP, hexafluoroisopropanol; IC31, two-component adjuvant comprising KLK and ODN1a; IEX, ion exchange; IU, international units; KLK, 11-mer cationic peptide; component of IC31; MALDI-TOF, matrix-assisted laser desorption ionisation—time of flight; MSA, methanesulfonic acid; ODN1a, synthetic oligodeoxynucleotide; component of IC31; TEA, triethylamine; TEAA, triethylammonium acetate; TFA, trifluoroacetic acid; Tris, tris(hydroxymethyl)aminomethane

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the compound using defined chemical-analytical methods. In addition, these methods should support stability studies. It is well established that cationic molecules and anionic peptides/oligonucleotides form ionic complexes [11–13]. These complexes are mainly formed by ionic interactions leading to poorly water-soluble or insoluble polyelectrolyte complexes [14–16]. Therefore, quantitative analysis of both components is very challenging. In some cases where the complex is caused by weak ionic interactions, sample preparation using physiological salt conditions is sufficient [17]. On the other hand, for separating complexes, which are caused by ionic, hydrophobic and other unknown interactions, the above mentioned sample preparation would be insufficient.

The aim of the studies described here was to establish a separation technique for IC31 to make the identification and quantification of the cationic peptide and the oligodeoxynucleotide by reversed phase HPLC possible. Furthermore, such methods should be useable for stability studies.

2. Materials and methods

2.1. Chemicals

Lyophilized heparin (sodium salt from porcine intestinal mucosa; minimum 140 USP/mg) was obtained from Sigma (Sigma–Aldrich Austria/Germany). Triethylamine (TEA), 3-hydroxypicolinic acid (HPA), low molecular weight dextransulfate (MW ∼5.000) and methanesulfonic acid (MSA) were from Fluka (Sigma–Aldrich Austria/Germany). Glacial acetic acid and 1,1,1,3,3,3-hexafluoroisopropanol (HFIP) were purchased from Merck (Darmstadt, Germany). Acetonitrile was obtained from Fisher Scientific (Fisher Scientific UK Limited; UK). Trifluoroacetic acid was obtained from Pierce (Rockford, IL). Water was purified in-house using a Milli-Q system (Millipore, Bedford, MA). High molecular weight Dextransulfate MW ∼500.000 was obtained from Pharmacia (Pharmacia, Sweden). All reagents were of analytical grade if not stated otherwise.

ODN1a, a custom made oligodeoxynucleotide with a normal phosphate backbone and with the sequence 5′-ICI-CIC-ICI-CIC-ICI-CIC-ICI-CIC-IC-3′, was synthesized by Proligo (Boulder, CO). KLK, a custom made peptide with the sequence NH_2-KLKLLLLLKLK-COOH, was synthesized by MPS (Multiple Peptide Systems; San Diego, CA).

2.2. HPLC equipment

The Alliance 2695 separation module with a dual wavelength detector (2487 Dual λ Absorbance detector; Waters Corp., Milford, MA) was used for analytical HPLC experiments. The oligodeoxynucleotide was analyzed using a 3.9 mm × 20 mm XTerra® RP_{18} guard column and a 3.9 mm × 150 mm XTerra™ RP_{18} column, both packed with 5 μm particles (average pore diameter 124 Å; Waters Corp. Milford, MA). The peptide was analyzed using a 4 mm × 200 mm Suplex PKB-100 column packed with 5 μm particles (average pore size 120 Å; Supelco; Bellefonte, PA).

Beside acetonitrile, 20 mM MSA and 100 mM HFIP/2.04 mM TEA were used as eluents.

2.3. UV–vis experiments

UV–vis experiments were carried out to observe the dissociation of the peptide/ODN complex under various conditions.

A stock solution of the oligodeoxynucleotide in water was diluted to a concentration of 20–25 μg oligodeoxynucleotide/ml either with water or heparin in various concentrations and at various pH values.

A wavelength scan was performed in all experiments between 200 and 400 nm and data were printed in a 20 nm interval (Hitachi U-2001 Spectrophotometer). The spectra were printed to compare them visually.

2.4. Stability studies of ODN1a and KLK

To record the breakdown of the ODN1a molecule, samples were analyzed by MALDI-TOF. In parallel analyses by RP-HPLC were carried out.

Sample preparation for MALDI-TOF analyses was done similarly as described by Ball and Packman [18]. In short, an aqueous ODN1a solution (1 mg/ml) was pipetted through a Zip-Tip C18 (ZTC18S024; Millipore) to bind the sample on the C18 material and to concentrate it. To elute ODN1a, matrix (75 mg/ml HPA in 50% acetonitrile) was picked up and the mixture was eluted directly onto the sample plate. To remove the salt adduct signals, 0.5 μl of IEX beads in water were added to the sample-matrix mixture by mixing it up and down in the pipet tip a couple of times. Negative ion spectra were acquired on a Voyager DE STR (PerSeptive Biosystems; Applera Handelsges., Austria) with a high laser intensity of (∼3100) in the linear mode.

Degradation of KLK was induced by the addition of trypsin, as this enzyme cleaves C-terminally of lysine and arginine. A KLK stock solution of 5 mg/ml in water and 100 μl of 0.25 μg/μl trypsin in water were prepared. The KLK solution was diluted as follows:

Eighty microliters of KLK stock were mixed with 16 μl of the trypsin stock and with 100 μl of 40 mM Tris/540 mM NaCl and made to 400 μl with water. The sample was incubated at 37 °C for 2 h. Every 30 min, 50 μl were withdrawn and made to 1 ml with 30% AcOH. Each sample was analyzed in duplicate by RP-HPLC.

3. Results and discussion

3.1. Dissociation and quantification of the IC31 complex

The development of a complex dissociation method and an analytical method was done in parallel. As the mixture of

Gradient of the peptide

min	ml/min	A (%)	B (%)
	0.8	90	10
12.0	0.8	40	60
12.5	0.8	5	95
13.5	0.8	5	95
14.0	0.8	90	10

Column temperature: 45ºC 5ºC;

injection volume: 50 μl

A: 20 mM MSA

B: acetonitrile

Gradient of ODN1a

min	ml/min	A (%)	B (%)
	1	99	1
1.0	1	99	1
11.0	1	89	11
12.0	1	60	40
15.0	1	60	40
15.5	1	99	1
19.0	1	99	1

Column temp: 50ºC 5ºC; injection

volume: 25 μl

A: 100 mM HFIP/2.04 mM TEA

B: acetonitrile

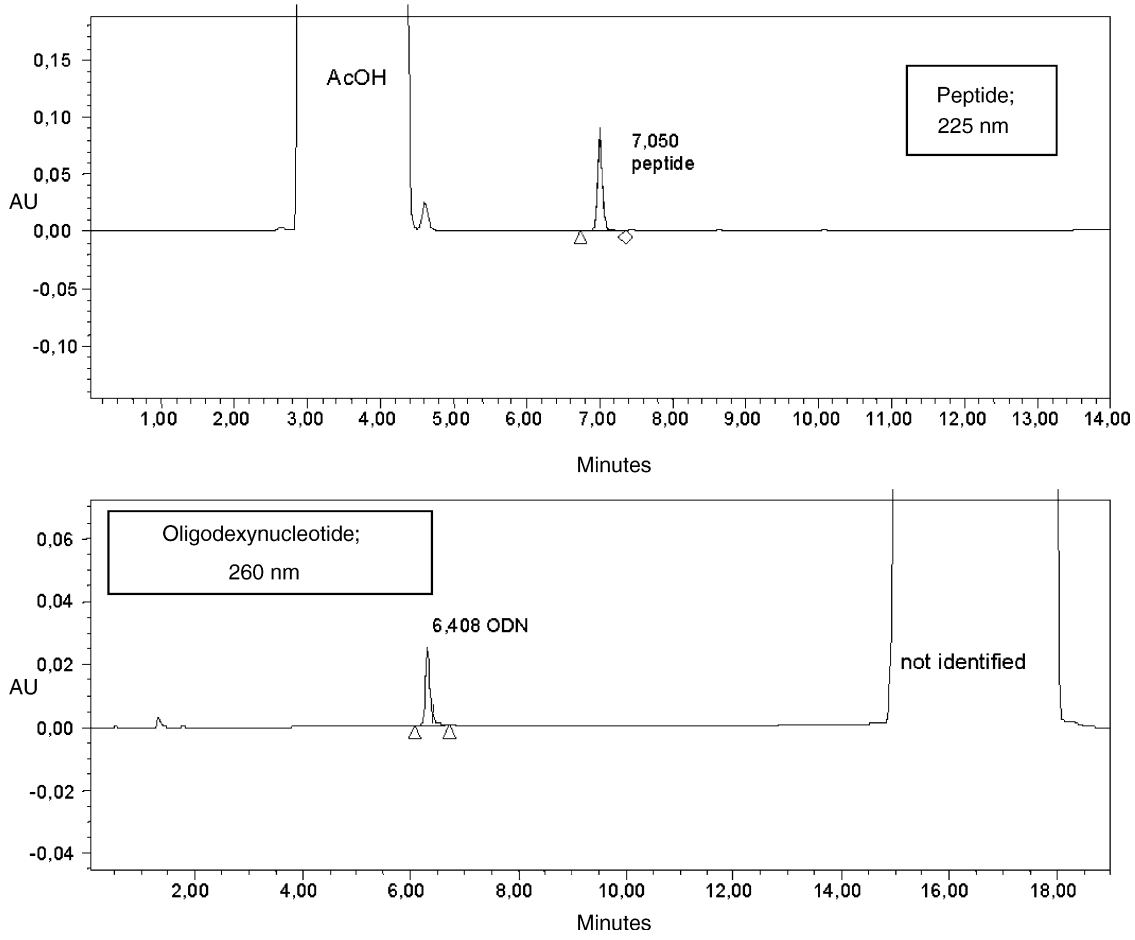

Fig. 1. The peptide and the ODN1a analysed in two separated runs. Sample preparation for the peptide: between 20 and 100 μg/ml dissolved in 30% AcOH. Sample preparation for the ODN1a: between 10 and 50 μg/ml dissolved in water. The peak between 3 and 4.5 min is acetic acid. The late eluting peak in ODN1a (15–18 min) has not been identified but also occurs in the blank run.

the peptide (KLK) and the oligodeoxynucleotide (ODN1a; mix = IC31) in various aqueous formulations is a suspension, the formation of a water insoluble complex is apparent. Hence, the dissociation of the complex is necessary for quantitative analysis of both components. The development of an analytical method was performed in two steps. First, we aimed at developing a method to determine both components in a single HPLC run. Different combinations of eluent systems were used and various ion-pairing buffer systems taken from published data were tested to evaluate the elution behavior of the ODN1a [19]. It was found that about 10% acetonitrile were sufficient to elute ODN1a regardless of the aqueous eluent. As mobile phase for KLK a mixture of 0.05% TFA in water and 0.05% TFA in acetonitrile was chosen because of the good resolution seen with this system. The sample preparation of IC31 formulations was performed with 0.1 M HFIP to dissociate the complex.

After testing various eluents, gradients as well as changing the elution order of the two components, it became obvious that the complex could not be quantified in a single HPLC run (data not shown). Furthermore, during these experiments it became apparent that the oligodeoxynucleotide used is pH sensitive. Traces of acid were sufficient to degrade the molecule leading to a lower than expected AUC at the same retention time (see chapter "stability indicating properties"). Hence, the analysis of both components in one step was discontinued.

To analyze IC31 in two separated runs, the sample preparation for analysis was changed. IC31 was diluted to concentrations of 50–100 μg/ml with 30% acetic acid prior to the analysis of KLK. This sample preparation had the added advantage that the oligodeoxynucleotide was degraded by the loss of purine bases (see Section 3.2) in the same step and no additional peak of ODN1a residues occurred in the chromatogram (see Fig. 1). Eluent A was changed to 20 mM MSA, due to an improved elution profile of KLK. Control samples with the same concentration of KLK but without the oligodeoxynucleotide and prepared in the same way for HPLC analysis as IC31 samples, showed no significant difference in response factors. This ODN1a related independence was clearly shown in a HPLC method validation program for KLK as part of IC31, as the accuracy for this HPLC method was also proven for analyzing IC31. An average peak area recovery of 98.5% (±1.8%) was determined with nine different formulations covering three different concentrations (data not shown). The linearity of this method was shown for a range of 0–150 μg/ml KLK. This range covers a broad spectrum of possible KLK concentrations in IC31 formulations.

The gradient of ODN1a was adapted from previous experiments but shortened to 19 min (see Fig. 1). To improve the elution of ODN1a eluent A was changed again, by raising the concentration of HFIP to 100 mM and decreasing the concentration of TEA to 2.04 mM. ODN1a alone prepared in water gave a sharp peak at 8–10% acetonitrile. Consecutive blank runs did not show any peak of ODN1a indicating that no material was retained on the guard or main column.

However, the second part of the ODN1a analysis – releasing ODN1a from the IC31 complex – was not achieved. 0.1 M HFIP was clearly insufficient to dissociate KLK and ODN1a.

As mentioned in the introduction, physiological salt conditions are sometimes sufficient to release anionic compounds from weak ionic complexes [17]. Considering the number of positive charges in the peptide a higher molarity of various salts was tested. Hence, further experiments were carried out with 3 M NaCl, 3 M Na-acetate, 6 M guanidine-HCl and 6 M urea to separate the peptide/ODN1a complex. Samples were prepared with the above-mentioned solvents and analyzed as described before. However, none of these components led to the quantitative dissociation of the IC31 complex.

It is well established that large sulfated anionic polymers such as dextran sulfate or heparin are able to release oligonu-

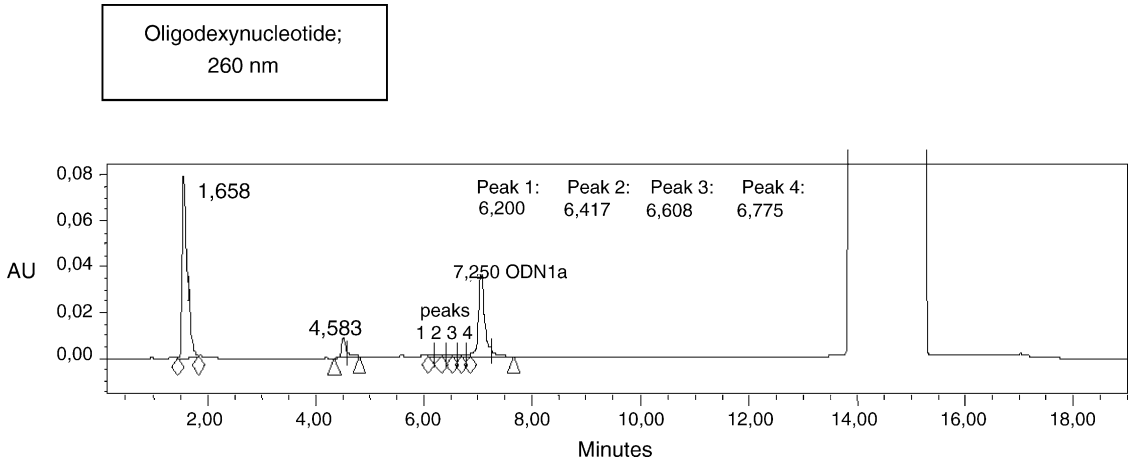

Fig. 2. Degradation products of ODN1a in acidic milieu. After incubation with 0.1M HCl for 270 min. a clear degradation of the oligodeoxynucleotide was observed. Additional peaks were detected in front of the main peak and also the characteristic injection peak (1.66 min) increased significantly compared to intact ODN1a.

cleotides and DNA from ionic complexes [11,20]. It was shown that dextransulfate and heparin are capable of displacing oligonucleotides from a complex of positively charged liposomes in a charge ratio-dependent, time dependent but also pH-dependent manner [11]. Although cationic lipids are a different class of compounds, the physical properties are similar (e.g. cationic and hydrophobic) and therefore, a similar behavior towards polyanions might be expected. Based on these findings a new set of experiments was designed to achieve the dissociation of the peptide/oligodeoxynucleotide complex with these large, highly anionic molecules. Tests with dextransulfate either of low molecular weight (∼5.000) or high molecular weight (∼500.000) did not give complete dissociation (data not shown). Increasing the amount of high molecular weight dextransulfate from 1 to 5 mg/ml only improved the recovery of ODN1a from 60 to 80% compared

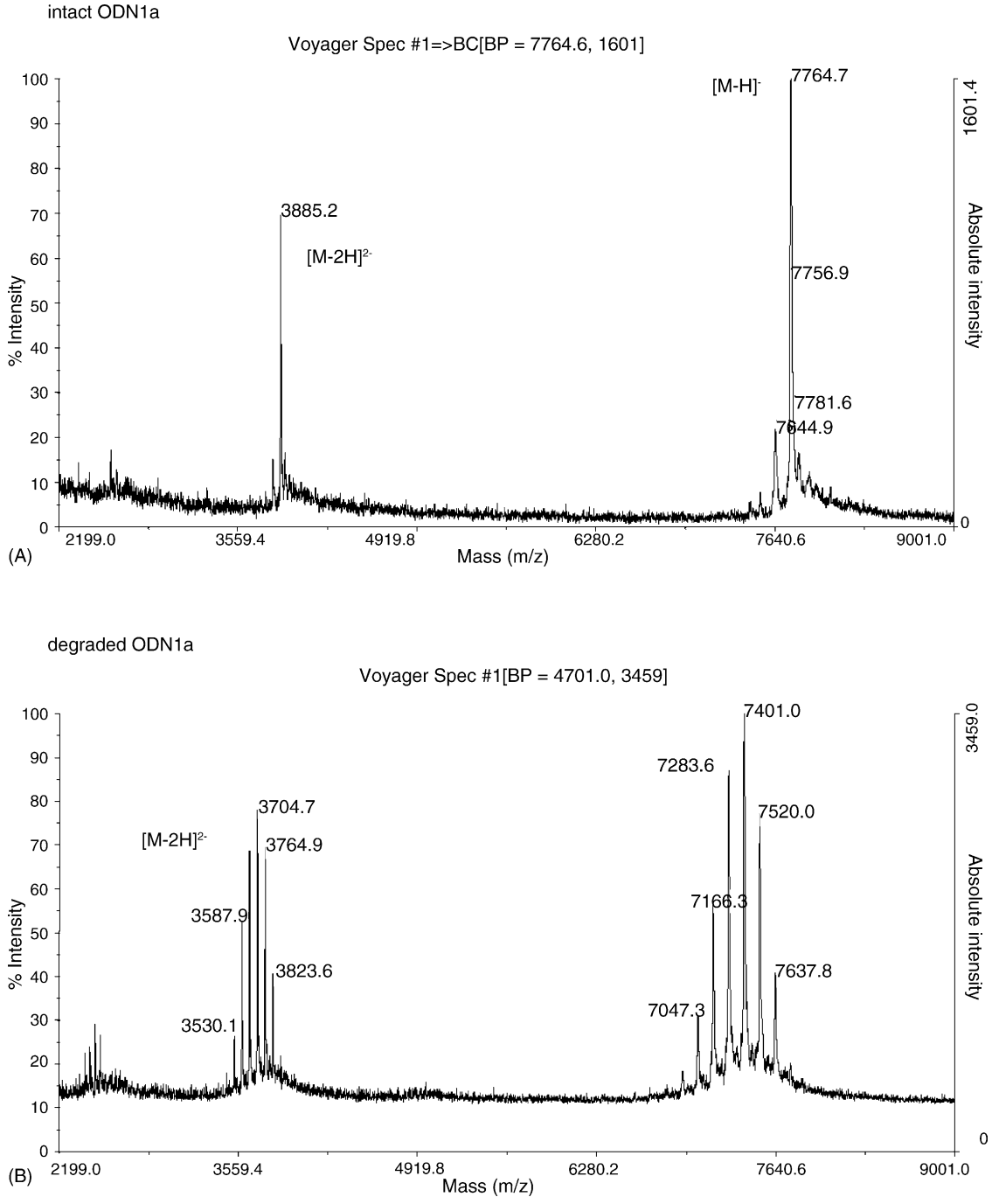

Fig. 3. MALDI-TOF analysis of ODN1a and degradation products. Comparison of intact (A) and degraded (B) ODN1a; The degradation profile was obtained after 270 min. incubation in 0.1 M HCl at room temperature. Significant degradation is apparent. The difference between two peaks corresponds to the loss of inosine (Δ = 119 Da).

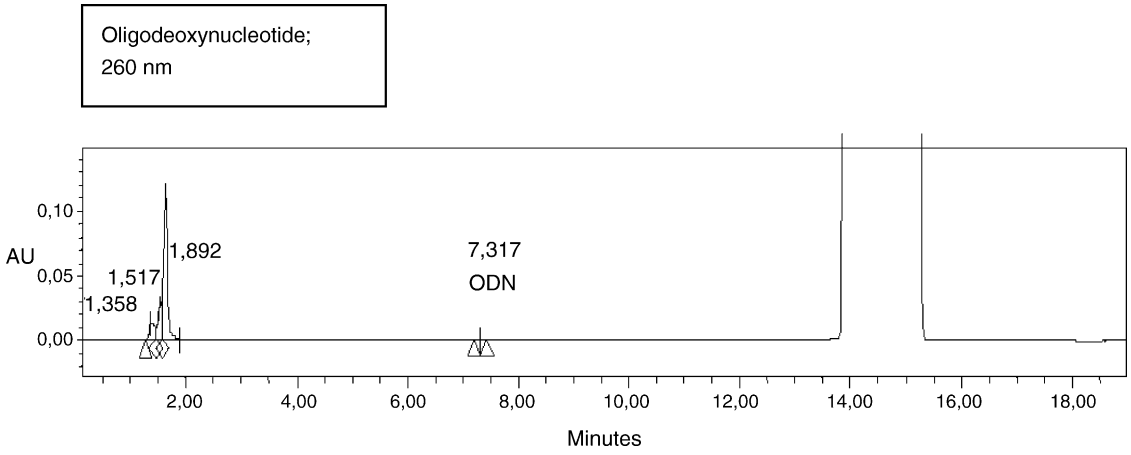

Fig. 4. HPLC analysis of degraded ODN1a. Chromatogram of ODN1a sample treated with 0.1 M HCl and 1 M NaOH. The AUC and height of the peak eluting in the void volume increased significantly compared to a chromatogram of an untreated ODN1a. The original peak of ODN1a after ∼7.3 min is hardly detectable.

to ODN1a in water (data not shown). Even higher amounts of dextransulfate were insufficient to achieve close to 100% recovery. Using low molecular weight dextransulfate, no dissociation of the complex was observed at all.

Next, different concentrations of heparin at various pH values were used. The main mechanism of its anticoagulation effect is thought to be the binding to lysine in antithrombin, inducing a cascade of reactions leading to the prevention

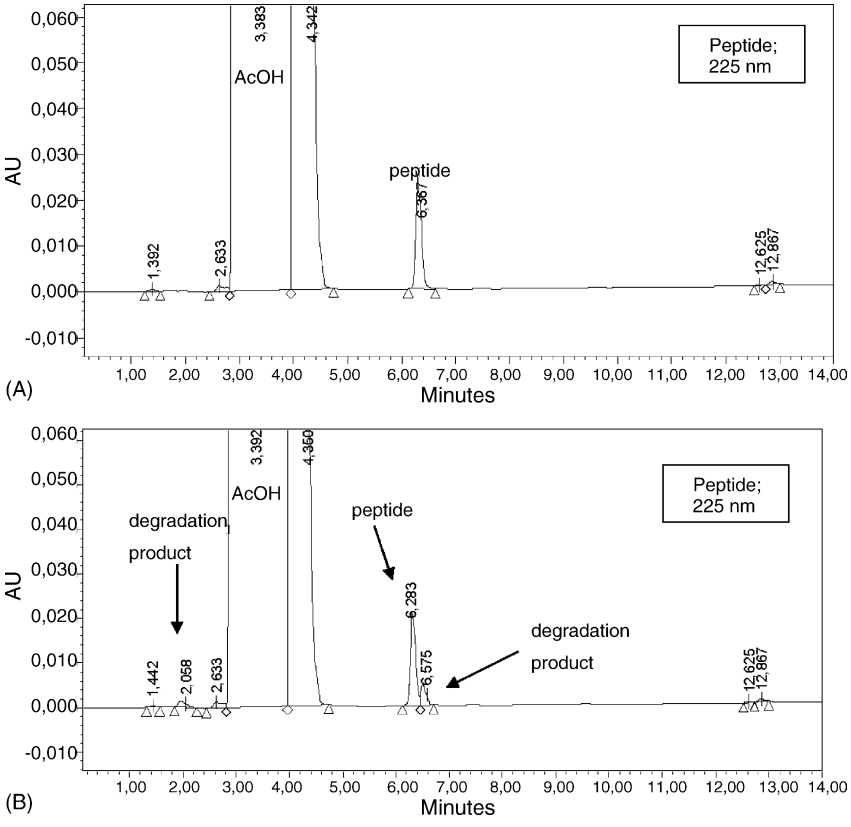

Fig. 5. Chromatograms (A–D) show degradation of KLK by trypsin as example enzyme. (A) At time 0; typical chromatogram of KLK in an aqueous matrix. Several small peaks in front the injection peak and at the end of the run derive most likely from impurities of the used materials. (B**)** After 30 min: A second peak after ∼6.6 min is already visible. Another peak after ∼2.06 min in front of the injection peak (AcOH) occurs too. The AUC/height of the main peak is significantly smaller than 30 min before. (C**)** After 60 min: The peaks after ∼2.1 and ∼6.6 min are significantly increased whereas the main peak is significantly smaller than before. (D) After 120 min. the AUC/height of the degradation peak after 6.6 min is already almost identical with the main peak of the peptide. The peak after ∼2.1 min (in front of the injection peak) stayed constant.

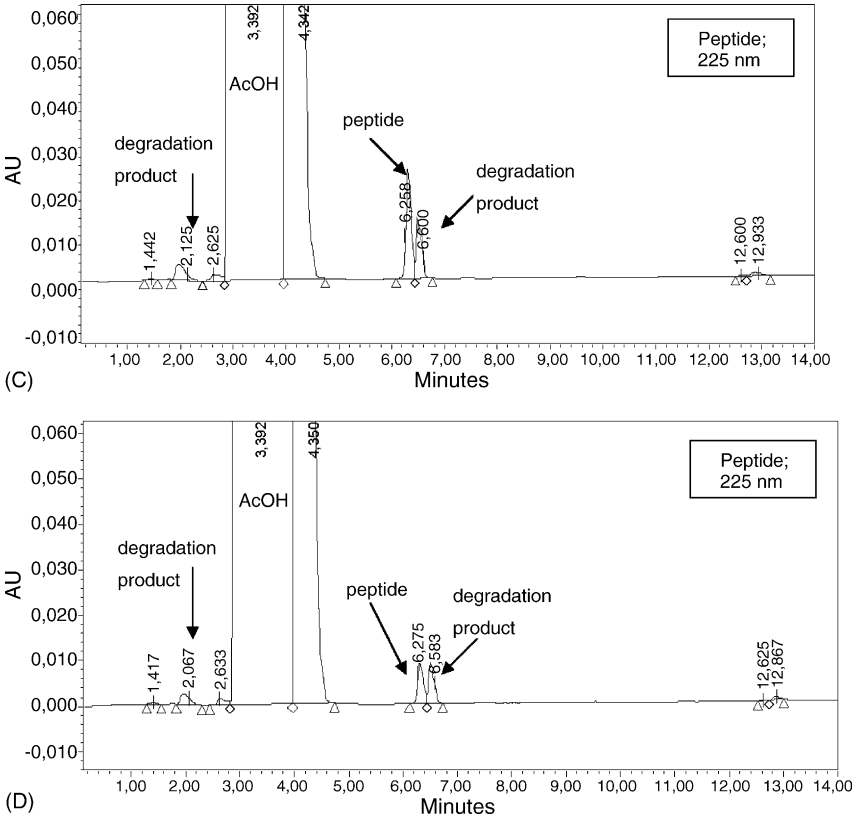

Fig. 5. (*Continued*).

of blood clotting [21]. This physical property turned out to be relevant for the dissociation of the IC31 complex. Measuring the absorption of different ODN1a/heparin mixtures by UV–vis (see Section 2.3) showed that the ODN1a specific absorption increased by raising the pH stepwise from 3 to 12 and increasing the concentration of the heparin solution from 250 to 500, 750 or 1000 IU/ml. The absorption of the heparin solution was not influenced significantly by the change of the pH but slightly increased at higher concentrations (data not shown). The chromatogram looks the same as for ODN1a in water (see Fig. 1) in regard to retention time and peak shape. Only the peak area is slightly different due to the absorption of heparin. RP-HPLC analysis of these samples showed degradation products as depicted in Fig. 2, using a heparin solution with a pH lower than 7 for IC31 sample. A reproducible recovery rate of 100 ± 5% was only achieved by using a heparin solution of 1000 IU/ml with a pH of 11–12. A lower concentration of the heparin solution, such as 250, 500 IU/ml or even 750 IU/ml led to dissociation of the IC31 complex but the recovery rate was below an acceptable value, i.e. 95%. Hence, 1000 IU/ml and a pH of 11–12 were considered as the best sample conditions for ODN1a analysis in IC31. This sample preparation technique was proven to be valid in a detailed HPLC method validation program for ODN1a as part of IC31. The linearity of this method was tested between 0 and 25 μg/ml, but also higher concentrations (up to 75 μg/ml) are in a linear range. The peak recovery rate of ODN1a in an IC31 formulation was shown to be in a range of 99.5% (±1.5%) when testing the accuracy of the method.

3.2. Stability indicating properties

As already mentioned before, both HPLC methods were also used to monitor degradation products. First, experiments with aqueous ODN1a solutions in alkaline or acidic milieu were carried out. The method parameters of the analytical method used for monitoring degradation by reversed-phase HPLC were slightly different compared to the final method as described in the table of Fig. 1. The gradient started with 100% of 100 mM HFIP/2.04 mM TEA instead of 99%, but showed the same steepness. The reason was to observe as many degradation products as possible. As shown in Fig. 2, treatment of ODN1a with 0.1 M hydrochloric acid for 150 min resulted in additional peaks in the chromatogram. With prolonged incubation time (270 min) these additional peaks increased in area with a concomitant decrease in the peak area of the intact ODN1a (data not shown). A MALDI-TOF spectrum of this sample was recorded after 270 min (Fig. 3). The degradation peaks were smaller in mass than the original one and showed the same m/z difference between each other, i.e. 119 Da. Based on the ODN1a sequence the mass difference of 119 can be explained by the loss of inosine from the backbone. The backbone of the ODN1a was

still stable after treatment with 1 M HCl, as the main ODN1a peak still showed the same retention time in the HPLC chromatogram as before the treatment. The break of the backbone was only achieved when the pH of the acidic ODN1a solution was raised again to a pH above 8. (Fig. 4). As indicated in the chromatogram the main peak is hardly visible whereas the peak of the void volume increased significantly. On the other hand, incubation with 1 M NaOH without acidic pretreatment at room temperature or even at temperatures above 50 °C did not affect the stability of the ODN1a. This finding played also an important role for the development of the sample preparation for ODN1a analysis. Degradation of the ODN1a by the alkaline heparin solution can be excluded.

Incubation of KLK in either alkaline or acidic milieu at ambient temperature did not result in any degradation. Even increasing the temperature did not initiate the degradation process. These results confirmed previous experiments where it was observed that KLK is rather insusceptible against the variation of the pH but very sensitive against several proteases. To show typical degradation products of KLK caused by enzymatic activity, the peptide was treated with trypsin. KLK in a concentration of 1 mg/ml was incubated with trypsin (10 μg/ml) at pH 7.5 (see Section 2.4). Every 30 min samples were withdrawn and analyzed by reversed-phase HPLC (see Fig. 5A–D). As shown in Fig. 5 B, after 30 min, already a very dominant peak occurred in the void volume and a second peak, not baseline separated, was observed after the parent peptide peak. As expected the AUC of the degradation peaks increased time-dependently. After 2 h (Fig. 5 D) the most prominent degradation peak reached almost identical height and AUC as the original peptide peak. As a result of these degradation studies both analytical methods were considered as stability indicating.

4. Conclusion

To guarantee the quantitative analysis of IC31 the complex of KLK and ODN1a has to be dissociated first. The sample preparation of KLK with 30% AcOH is sufficient to achieve an appropriate sample preparation for reversed-phase HPLC analysis regardless whether KLK is associated with ODN1a or not. Furthermore, the sample preparation is independent from the presence of ODN1a. In comparison, the sample preparation for ODN1a is different whether a cationic compound is present in the formulation or not. Without peptide water is sufficient for HPLC sample preparation of ODN1a, whereas heparin and a basic pH are required to release the oligodeoxynucleotide from the complex for quantitative analysis.

Experiments showed that both analytical methods have stability indicating properties. All these findings led to the conclusion that both HPLC methods and the according preparation procedures can be employed as routine methods for release testing.

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