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Journal of Chromatography A, 1079 (2005) 397–407
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

Studies on the use of needle-free injection device on proteins

Kálmán Benedek[a,*], Ekaterina Walker[b], Lonnie A. Doshier[a], Richard Stout[b]

[a] *iGORi, 1543 N. Moorpark Road No. 319, Thousand Oaks, CA 91360, USA*
[b] *Bioject Inc., Portland, Oregon, USA*

This paper is dedicated to the memory of Professor Csaba Horváth, the "father of HPLC", a friend, a mentor, an exceptional scientist and a true renaissance man.

Abstract

In the following communication we report the evaluation of 18 proteins that were processed by a specific needle free injection device. The processed protein samples were analyzed by two HPLC techniques, reversed-phase liquid chromatography (RPLC) and size-exclusion chromatography (SEC). These techniques are two of the most widely used analytical techniques in the biopharmaceutical industry for the characterization, integrity assessment and stability study of peptide and protein products. The results indicate that needle free injection, using the specific device of this study, is not damaging to the studied proteins and does not generate aggregates. We found no evidence of the predicted possible effects of needle free injections, and concluded that needle free delivery is in general not different than any other delivery system and that its use should be evaluated on a case by case basis. It has to be noted that there are various needle free device designs and our work was performed using an Iject® from Bioject. Our conclusions therefore should be limited to the Iject® design we used in this study. In the reported experiments we used commercially available (economical) model proteins, which facilitate the use of the results for future comparison and reference. The work reported here can serve as a reference to illustrate the benign nature of our needle free injection device. It also highlights an interesting analogy between a set of phobias that were seen to have plagued the early stages of biochemistry and HPLC, on the one hand, and some attitudes that appear to hinder the widespread acceptance of needle free injection at present time, on the other. These phobias were identified and named by Professor Csaba Horváth, the father of HPLC, as barophobia, siderophobia and lithophobia. Today a wealth of evidence is available to indicate that those phobias are ungrounded and that the negative observations can be explained in most cases by adsorption and prevented by proper formulations and solvent conditions.

Keywords: Needle-free injection; Protein stability; Protein HPLC; Pressure injection; Biopharmaceuticals; Drug delivery

1. Introduction

Needle-free injection technology has been used in clinical practice for many decades and has been shown to be safe and effective for the administration of many different medications for a variety of applications, including immunization and mass inoculation of large populations [1–9].

Needle free injection based delivery systems for the delivery of peptide and protein based biopharmaceuticals are becoming increasingly popular for a number of reasons. One of them is that the method eliminates the fear of injection, which can involve *diatrypophobia*, the fear of piercing, or *belonephobia*, the fear of needles generally. Patients such as diabetics who need to inject themselves on a regular basis are very much in favor of this comfortable delivery method. However, one of the major roadblocks for the more general use of needle free injection is that only limited information is available in the public domain on the effects of needle free injection on the integrity of peptides and proteins. The reason for this is due partially to the novelty of this delivery system, as well as partially to misinformation about possible negative effects on biopharmaceuticals. It is telling that previously used names like jet injection or pressurized injection

* Corresponding author. Tel.: +1 818 5942023; fax: +1 707 2811324.
E-mail address: kbenedek@igori.com (K. Benedek).

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doi:10.1016/j.chroma.2005.03.098

have been eliminated to minimize the negative perceptions those names created. Current knowledge of the benefits of this drug delivery system is limited to a select few scientists within the pharmaceutical industry, and the details of the experimental results they generated have been shielded by confidentiality agreements.

The essence of needle free injection is that the pharmaceutical drug product is injected through the skin by pressure instead of by a traditional needle and syringe method [10,11]. The benefits of this unique delivery system are numerous. It is convenient, physiologically accepted, fast, no sterilization of the device is required and most importantly it is ideal for the delivery of peptide and protein pharmaceuticals [12,13]. One of the great benefits of needle free delivery is that biopharmaceuticals delivered by needle free injection do not have to pass the gastrointestinal system and its degradation industry [14–18]. The opportunity to have biopharmaceuticals delivered more efficiently can thus prove extremely beneficial or in some cases may be the only viable option.

However, the widespread implementation of any new device, technology or even industry often invites rejection based on our previous experience, which is too often influenced by prejudices or phobias.

Most researchers would agree that there exist three major fears (phobias) based on long standing scientific reflexes that come into play with any type of protein handling.

(1) The proteins' primary structure could be affected due to degradations (primary structure).
(2) Irreversible conformational changes could occur (secondary and tertiares structures).
(3) Protein aggregation also could occur (quaternary structure).

In the case of needle free injection one of the most often used arguments is that the injection can damage a protein. The potential effects can depend on the combination of applied pressure, flow path design and the material characteristics of the nozzle.

It is interesting that similar fears surfaced in the early days of protein HPLC and initially hindered the wide acceptance of HPLC in the field of peptide and protein separation. Biochemists were concerned about the effects of pressure, flow through small pores and the materials used in HPLC on the integrity of peptides and proteins.

Because of these apparent similarities we feel justifying to reminding ourselves of those concerns and the way they were handled. Some of those fears were named by Professor Csaba Horváth as *barophobia*, or the fear of pressure, *lithophobia*, or the fear of cuts, which could occur when protein solution is forced through the pores of the porous silica beads, and *siderophobia*, or the fear of any iron containing transport line which could cause damage to proteins. These fears were ultimately resolved by thoroughly executed experimental research and over a few years it became obvious that in general the fears were unfounded. When in some individual cases problems did surface, they were shown to be more characteristic to the individual proteins than to the technique itself. Detailed studies helped to clarify the problems and in most cases these were not related to the defined phobias, but were rather the results of the presence of surfaces, and they were overcome by appropriate formulations and solvent conditions.

The methods selected for this study on the effect of needle free injection on proteins are aimed at following changes in the primary and quaternary structure of proteins. The fundamental concern is about degradation and aggregation, changes that are most devastating if they occur during any delivery method. Other changes should be studied separately and are beyond the objectives of this study.

It is ironic that in this work we use an analytical technique, HPLC, to resolve some of the phobias related to needle free injection, which itself had to overcome the same phobias in the 1980s [19–21].

2. Materials and methods

2.1. Chemicals

All proteins and phosphate buffered saline (PBS) were from Sigma (St. Louis, MO, USA). The chromatographic solvents, 1-propanol and trifluoroacetic acid (TFA) were purchased from Fisher (Pittsburgh, PA, USA).

2.2. Chromatographic conditions

The RPLC column was an SB-300 C8 from Agilent (Wilmington, DE, USA). Column dimensions are 150 mm × 4.6 mm and it is packed with 5 μm StableBond silica particles with 300 Å pore size. We employed gradient elution using 0.1% TFA in water as A solvent and 50% 1-propanol and water containing 0.1% TFA as B solvent. The linear gradient ascended from 5 to 50% B solvent. The flow rate was 0.5 mL/min. The elution of proteins was detected at 215 and/or 280 nm.

The SEC column, TSK G2000SWXL was from Tosoh Biosciences (Montgomeryville, PA, USA). We used 2× PBS as mobile phase, 0.5 mL/min flow rate and the elution was detected at 215 or 280 nm.

2.3. Needle free injection device

The Iject was from Bioject (Portland, OR, USA) is a prefilled single-use disposable injection device configured to administer 0.5–1.00 ml subcutaneous or intramuscular injections. The device is distributed "ready to use". Thus, it requires no additional parts or modifications for function. The device is activated by rotating the trigger sleeve 180°, and an injection is administered by advancing the trigger sleeve while the nozzle is held against the injection site. The Iject needle-free injection system is an investigational device, subject to the US Food and Drug Administration clearance for commercial distribution.

2.4. Experiments

All proteins were prepared at 1 mg/mL concentration in water unless stated otherwise and a single stock solution was used for needle free injections. The processed samples were prepared as follows; the protein solution was transferred into the glass insert of the needle-free injection device and the solution was injected into glass vials. We used two controls, one was a needle–syringe control and the other contained the original, unprocessed protein solution; the latter was the HPLC standard. The needle–syringe control was prepared by transferring the protein solution from a syringe through a 22-gauge needle (394 μm) into a glass vial. In some cases the protein concentration was less than 1 mg/mL. Alterations from the original protocol will be noted.

All samples were transferred to HPLC sample vials and analyzed by reversed liquid-phase chromatography (RPLC) and size-exclusion chromatography (SEC).

3. Results

The model proteins were selected based on commercial availability, biochemical interest, and diversity in size, function and structure. The proteins studied in this report are all commercially available and as such can be used for comparisons in the future. Some of the proteins are similar to each other or derivatives of each other. The goal was to represent a broad spectrum of proteins by size, structure and function. Table 1 displays the list of proteins used in this series of studies.

Table 1
List of all the protein samples used in these studies

Number	Name	Abbreviation	MW
1	Cytochrome *c*	CYT	12300
2	Growth hormone releasing factor	GHRF	5108
3	Bovine serum albumin (monomeric)	mBSA	Monomer 66400
4	Bovine serum albumin (normal)	dBSA	Dimer 132800
5	Hemoglobin	HGL	∼68000
6	Immunoglobulin G (human)	hIGG	∼150000
7	Lysozyme	LYS	14388
8	Myoglobin	MYO	17000
9	Papain	PAP	23000
10	Somatostatin	SOM	1637
11	Thyrotropin releasing hormone	TRH	362
12	α-Chymotrypsin	CHY	25000
13	α-Chymotrypsin-PEG	PEG-CHY	25000
		PEG	∼45000
14	β-Lactoglobulin	BLACT	18300
15	Ribonuclease A	RNA	13700
16	Ribonuclease B	RNB	14700
17	Insulin	INS	5808
18	Lactic dehydrogenase	LDH	136700

It has to be noted that the elution conditions were not optimized for each protein separately. We used standard chromatographic conditions regularly used at iGORi for a number of protein projects. However, we felt that the use of general methods is necessary for such a large screening project. Since we did have appropriate controls present in the series the contributions of needle free injection and chromatography can be deconvoluted. In some cases the chromatographic conditions were inappropriate for selected proteins and those data were dismissed from the conclusions. Some of the difficulties will be noted below.

3.1. Evaluation criteria

We prepared a single stock solution from each protein, which we then used in three different ways as follows; three (or two) aliquots were processed by the needle free injection device (Iject), one by the needle and syringe method, and one aliquot was unprocessed and transferred directly into the HPLC sample vial and served as the HPLC control sample. Since the origin of all samples is the same, but the sample treatment is different, they could be treated as "parallel" samples.

Criterion 1: In case of parallel samples the chromatographic profile should look exactly the same for all samples regardless of the sample treatment. If a new peak would emerge in any chromatogram of the same protein sample that sample was probably affected by the experimental process.

Criterion 2: In case of parallel samples the integrated peak areas should be very reproducible. We arbitrarily decided that good reproducibility is defined by 5% relative standard deviation (RSD) of the peak areas of the same peaks from the "parallel" chromatograms. We usually injected five aliquots of the original protein solution, and if the processes had no effect on the proteins than the RSD of the calculated peak areas should be also around 5%. When the reproducibility is above 5% then the sample treatment as a whole probably affected the samples.

Criterion 3: In some cases we calculated the peak ratio of two peaks and defined that the RSD of the peak ratio for unaffected samples should be below 5%. This criterion was used to confirm sample integrity when the peaks of the chromatogram could not be identified due to a complex chromatographic profile. The identity of the peaks is not always necessary to show reproducibility since all peaks are originated from the original sample. To compare all peak areas could be a cumbersome exercise. The peak ratio of two selected peaks could be used for confirming reproducibility, a technique usually applied for peptide mapping. These criteria were used to evaluate both the RPLC and SEC data.

The proteins explored in these experiments were used as received. No extra purification was performed. In some cases we have multiple peaks in both RPLC and SEC for a "single" protein. The identification of the extra peaks was beyond the scope of this study. However, because we used standard conditions for the analysis for all of these proteins, if no

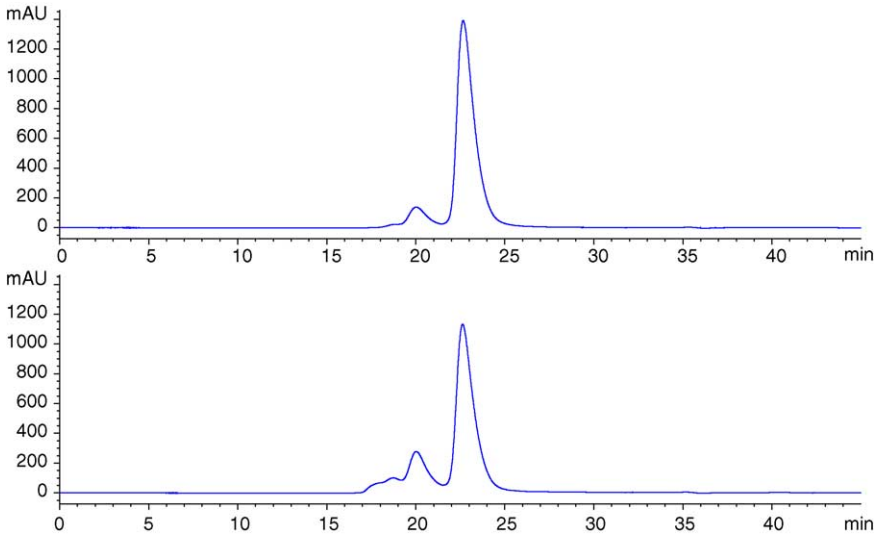

Fig. 1. SEC chromatograms of two BSA samples (monomeric and normal) at 215 nm.

degradation or aggregation happened, the chromatograms should be identical. As long as the chromatograms were "reproducible" the nature of the peaks is not critical. Some proteins however displayed anomalous characteristics and they will be mentioned as we report the specific results.

3.2. Aggregation studies

Albumin is known to have aggregates such as dimers, trimers and tetramers. We assumed that if needle free injection affects the state of aggregation of albumins then we would have more or less aggregates after needle free injection since the *seed of aggregation is* already present in all albumin samples. Two different types of bovine serum albumins (BSA) were used in this experiment, both at 1 mg/mL concentration. One of them is called "monomeric" and the other is called "normal" BSA. The "monomeric" BSA is not supposed to have any "meric" form of BSA. Fig. 1 displays the chromatograms of the two BSA samples, the upper panel of Fig. 1 is the "monomeric" and the lower panel contains the "normal" BSA. The "monomeric" BSA is supposed to contain only the BSA monomer (main peak), but the SEC analysis showed that it contains a significant amount of dimer (smaller peak), as is well illustrated in Fig. 1. The "monomeric" BSA however is depleted from the higher level aggregates.

Aggregation is usually a function of protein concentration. We prepared a 10 mg/mL solution of the "normal" BSA as well. The high concentration sample should show more markedly if there is any concentration effect involved with the aggregation–dissociation process of BSA. During the SEC analysis we injected 5 μL of the 10 mg/mL BSA instead of the usual 50 μL in order to have the same amount of protein on the column.

Table 2 lists the RPLC and SEC peak area results of all BSA experiments. We had five samples 1–3, Syringe, Reference are three needle free injections, a needle–syringe control and the unprocessed protein solution for each BSA solution, respectively. Table 2 also contains the average (AVE), standard deviation (SD) and relative standard deviation (RSD) of the peak areas.

Table 2
Peak areas of the five samples of each of the three different albumin solutions

	RPLC (280 nm)	SEC (215 nm)		
		I	II	III
BSA/M				
No. 1	3110	992	10896	97383
No. 2	3003	1032	10830	97781
No. 3	3016	909	10857	98589
Syringe	3061	918	10601	98492
Reference	3010	928	10511	98262
AVE	3040	956	10739	98101
SD	45	54	172	509
RSD (%)	1.49	5.61	1.60	0.52
BSA 1 mg				
No. 1	3092	9357	22652	81849
No. 2	3114	9661	22528	81096
No. 3		9611	22387	81011
Syringe	3126	9223	22155	81811
Reference	3141	8700	21512	80618
AVE	3118	9310	22247	81277
SD	21	386	450	536
RSD (%)	0.66	4.15	2.02	0.66
BSA 10 mg				
No. 1	3283	9512	21779	80173
No. 2	3244	9498	21748	80443
No. 3	3284	9434	21684	80606
Syringe	3345	9236	21479	80536
Reference	3305	9155	21335	80381
AVE	3292	9367	21605	80428
SD	37	162	191	166
RSD (%)	1.12	1.73	0.88	0.21

Based on the excellent reproducibility of the peak areas of the BSA dimers and monomers during the SEC analysis, we concluded that the needle free injection did not affect the "meric" distribution of the three BSA samples. No aggregation was observed for BSA and even at high protein concentration the monomer–dimer ratio did not change.

We also subjected the samples to RPLC and observed no differences in the chromatograms of the BSA samples, indicating that no degradation occurred.

The results clearly indicate that BSA did not change during needle free injections.

Table 3
RPLC Peak areas of cytochrome *c*, a prosthetic group containing protein

	Cytochrome *c*, RPLC 280 nm
No. 1	7781
No. 2	7891
No. 3	7882
Syringe	7920
Reference	7924
AVE	7880
SD	58
RSD (%)	0.74

4. Stability of prosthetic group containing proteins

Next, we studied cytochrome *c*, myoglobin and hemoglobin, proteins with prosthetic groups. Prosthetic groups are relatively rigid and they are embedded in the structure of the folded protein chain. Some of those groups can be released during conformational stress when they are not covalently attached to the peptide chain, as in cytochrome *c*. The effect of pressure on the structure of myoglobin was intensively studied [22–24].

Fig. 2 displays the RPLC chromatograms of myoglobin and hemoglobin. Both chromatograms contain two sets of peaks. In myoglobin and hemoglobin the heme groups are physically and not chemically immobilized in the peptide structure. The late eluting peak, in both chromatograms, corresponds to the heme-containing prosthetic group, which was confirmed by the spectral characteristics of the peak at higher wavelengths. The protein samples were used as received and no attempt were made to eliminate the free heme groups from the samples. The presence of the heme group could be the result of the RPLC conditions also since we see the free heme in the HPLC control samples as well. Myoglobin displays a single peak around 19 min while hemoglobin has two closely eluting peaks around 20 min; these peaks are corresponding to the globin chains [25,26]. The two early eluting peaks of hemoglobin probably represent the different peptide chains of the hemoglobin tetramers. During integration we combined the two peak areas into one. The chromatograms do not contain any other peaks and suggest that the protein structures are not chemically degraded. The ratio of the two sets of peaks is an indication of whether or not the structure of the heme groups is affected by the needle free injection.

The chromatogram of cytochrome *c* contains only one peak, eluting around ~14.7 min. Cytochrome *c* holds the heme group by covalent bonds. There is no indication of a late peak, which would correspond to the prosthetic group. The integration results are listed in Table 3.

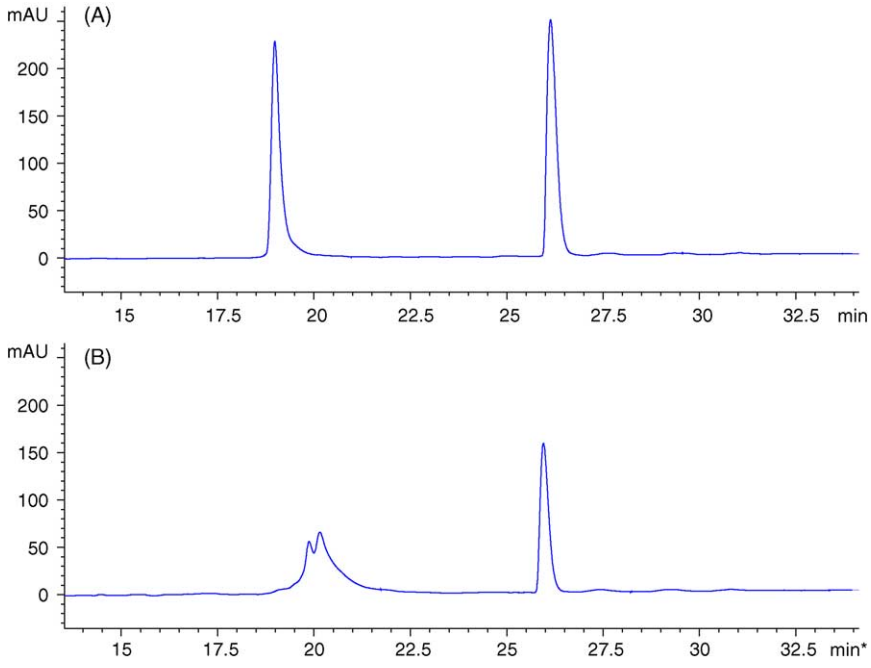

Fig. 2. RPLC chromatograms of myoglobin (A) and hemoglobin (B) at 280 nm.

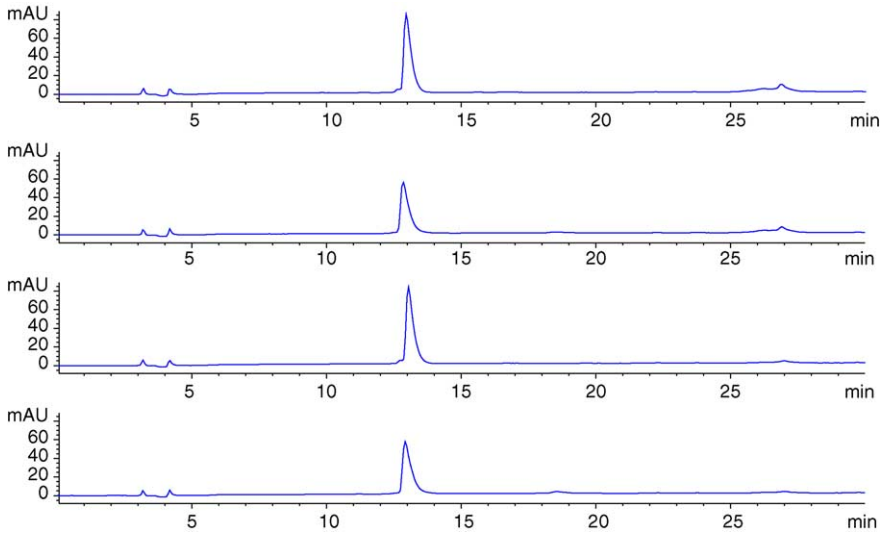

Fig. 3. RPLC chromatograms of ribonucleases A and B. The first and third panel from the top represents the needle free injected and the HPLC control sample of RNA. The second and the fourth panel from the top represent the needle free and the HPLC control sample of RNB.

The reproducibility of cytochrome *c* is excellent and no extra peak was observed on the RPLC chromatogram, indicating that cytochrome is stable during needle free injections.

The reproducibility of myoglobin peak areas is around 1.1% (RSD) indicating a very stable protein structure. The ratio of the two peak areas, which could be an indication of protein disintegration, is also very reproducible, providing evidence that the needle free injection is unlikely to affect the stability of these heme containing proteins.

The RSD of the RPLC peak area for the hemoglobin peaks is higher than 5%, which is our arbitrary definition of stability; this could be the result of integration error or could be an indication that some type of change could occur with the hemoglobin sample. However, the fact that the ratio of the two peaks is under 5% implies stability.

The SEC of myoglobin displays only one peak and the RSD of the peaks from the series is 1.14%, which is excellent in terms of reproducibility. Also there is no indication of a peak of substantially larger molecular component, which would indicate aggregation.

The SEC of cytochrome *c* indicates one small peak in all chromatograms at the expected elution volume of cytochrome *c*; however, the peak is very small and small integration error could result in a false conclusion in terms of reproducibility. However, it is clear that no peaks indicating large species were observed and the possibility of aggregation was dismissed based on these results. It is possible that there are some solubility issues, which interplay with SEC.

The SEC of hemoglobin displays two groups of peaks. The elution times of those peaks are relatively reproducible and their shape is also very similar. The peaks were not identified at this point; the similarity of the profiles of the chromatograms were convincing. Also we have not detected any peaks at earlier elution volumes on any of the chromatograms. Based on the above described results we concluded that needle free injection does not affect the selected prosthetic group containing proteins.

5. Stability of a glycosylated protein

We selected ribonucleases A and B for the comparisons. Ribonuclease A is the non-glycosylated form of ribonuclease B. The RPLC chromatograms are displayed in Fig. 3, while the SEC chromatograms in Fig. 4.

We did not observe any extra peaks on the chromatogram of the needle free injected sample as compared to the unprocessed HPLC standards, indicating that the peptide chains in both proteins are intact.

We also used evaporative light scattering detection following the elution of ribonucleases; the chromatographic profile was very similar to the UV profiles, and we did

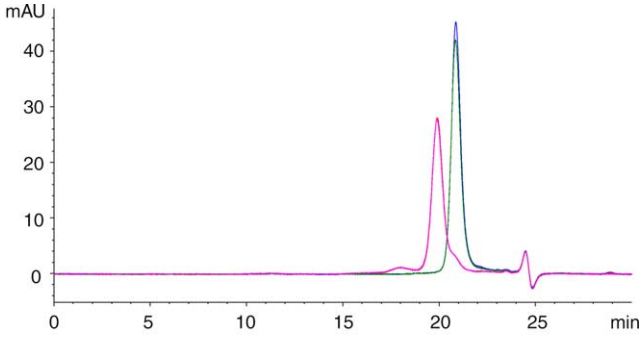

Fig. 4. SEC chromatograms of ribonucleases A and B. Two chromatograms of RNA and two of RNB are overlaid. One chromatogram is the needle free injection processed sample and the other is the HPLC control.

not observe extra peaks, which might be assigned to carbohydrate residues.

The earlier elution of RNB corresponds to a larger hydrodynamic radius of the molecule due to the carbohydrate residues. The SEC analysis of the ribonuclease samples is also very reproducible and there is no sign of any aggregation on the chromatograms.

6. Stability of a PEGylated protein

The next group of model proteins we studied to assess the effect of needle free injection consisted of the PEGylated and normal form of chymotrypsin. These model proteins are proteolytic enzymes and their chromatogram expected to be very complex due to self digestion. We in deed observed very busy chromatograms. The identification of all these peaks is beyond the scope of this study. However, according to our first criterion the concern is whether the chromatographic profile changes or not as a result of needle free injection as compared to the standards. It is demonstrated in Fig. 5 that the chromatographic profiles of very complex chromatograms of the chymotrypsin samples seem to be identical.

The RPLC integration results for chymotrypsin and PEGylated chymotrypsin vary very much. The reproducibility of the peak areas is very bad, around and over 25%, a clear sign of unexpected events. However, the peak area ratio of the selected peaks for chymotrypsin is relatively good (3.78% RSD) but the number (33.26% RSD) for PEGylated-chymotrypsin is very bad. These two proteins demonstrate the largest differences between the samples. The origin and the nature of the changes should be further studied. We will see similar complexity with the proteolytic enzymes later.

The SEC chromatograms of chymotrypsin display a major peak and the area of that peak is consistent and the process apparently reproducible. No aggregation products are visible on the chromatograms.

In the case of chymotrypsin-PEG, we have numerous peaks in the chromatogram; we selected the first eluting large peak and assumed it represents the whole PEGylated protein. The peak area again is remarkably reproducible and no aggregation could be observed.

Despite the very complex chromatograms of chymotrypsin and its PEGylated form we can assume that the needle free injection did not alter the general look of the chromatograms and consequently did not affect the molecules themselves either.

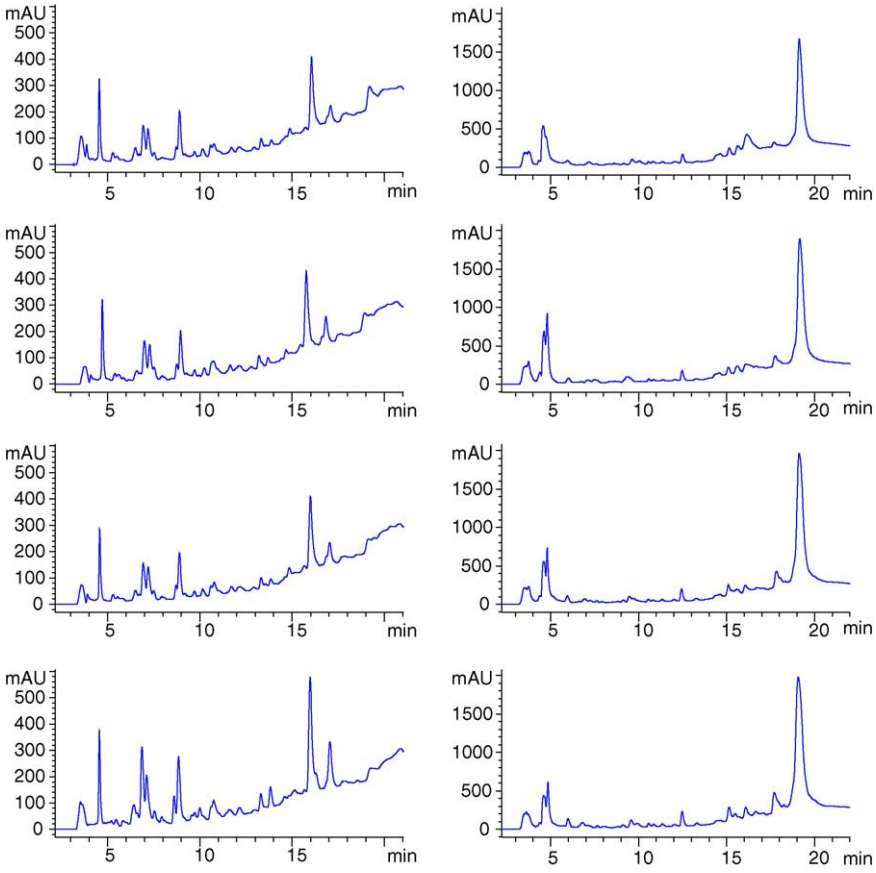

Fig. 5. RPLC of PEGylated chymotrypsin (first column) and chymotrypsin (second column) samples. The bottom row is the chromatogram of the needle and syringe standard. The chromatograms of the pressure-injected samples are in rows 1–3.

In order to really evaluate this protein much more controlled prepurified samples should be used, but for the purpose of this work the results are appropriate.

7. More single protein examples

Fig. 6 displays the RPLC chromatograms of selected single model proteins. We overlapped the five chromatograms of β-lactoglobulin and lysozyme in order to illustrate the remarkable similarity of the chromatograms. β-Lactoglobulin is composed of A and B isoforms, which only differ in two amino acids from each other. It has been shown that the pressure sensitivity of the two isoforms is different [27–32]. The midpoint of the pressure denaturation occurs at 123 MPa which is much higher than the ∼30 MPa value of the needle free injection and the pressure denaturation is also reversible [33]. No extra peaks can be noticed on the chromatograms of the five samples for any of the two proteins, indicating structural integrity to needle free injection. As we will show below, the peak areas are also remarkably similar.

Human immunoglobulin G samples display very similar chromatographic profiles. The IgG sample used in this experiment was polyclonal IgG, and the broad peak is consistent with such molecular mixtures. The similarity of the chromatograms is still remarkable and there is no indication of degradation.

The RPLC peak areas for β-lactoglobulin and lysozyme are reproducible within ∼2% and no extra peak can be observed. Table 4 displays the integration results for β-lactoglobulin and IgG. These data indicate that the proteins remained intact during pressure-injection.

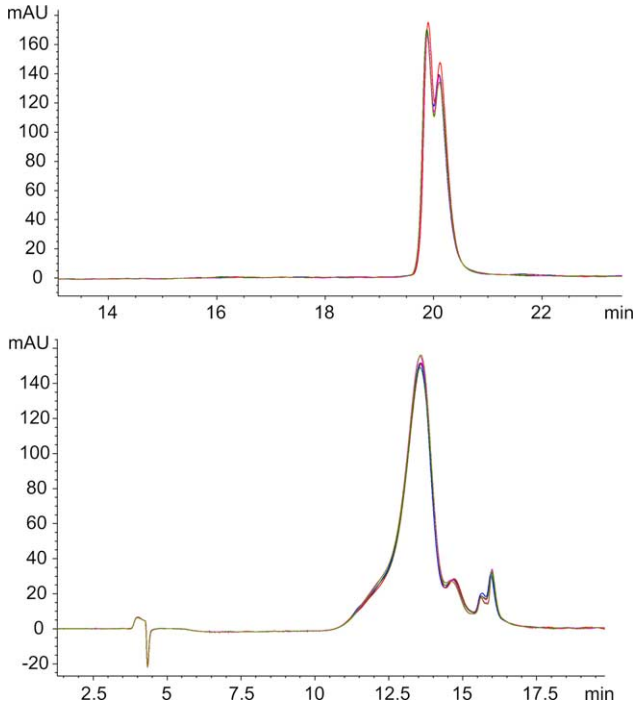

Fig. 6. Overlay of five RPLC chromatograms of lactoglobulin (upper panel), lysozyme (lower panel).

Table 4
Peak areas of β-lactoglobulin and IgG

	RPLC 280 nm	RPLC 215 nm	SEC 215 nm
Lactoglobulin			
No. 1	4414		102946
No. 2	4447		102983
No. 3	4422		103062
Syringe	4565		104558
Reference	4595		106882
AVE	4489		104086
SD	85		1703
RSD (%)	1.89		1.64
IgG			
No. 1		74784	155263
No. 2		84471	153165
No. 3		89958	152806
Syringe		90848	159885
Reference		95084	
AVE		87029	155280
SD		7819	3256
RSD (%)		8.98	2.10

It is interesting to note that the peak areas of the RPLC and SEC chromatograms of LDH using the same injection volume and detection wavelength are almost identical. The chromatographic profiles and the peak area reproducibility are excellent.

The slight change of the chromatographic profile of IgG could indicate some changes but not a significant degradation since no new peaks can be observed on the RPLC profile.

The SEC results for LDH, lactoglobulin and IgG indicate that no aggregation occurred.

8. Injection experiments with hormones and small peptides

The next group of samples included insulin, GHRF, somatostatin and TRH, which have molecular weights of 5808, 5108, 1637 and 362, respectively. This group represents the smaller molecules of the study. It is also relevant that bioactive molecules at this low molecular weight region could be the primary candidates for needle free delivery. A needle free delivery version of insulin is already on the market.

The RPLC and SEC profiles of the various insulin samples showed no indication of degradation or aggregation. The peak areas of the various samples are in very good agreement and also the peak areas of the RPLC and SEC peaks are almost identical when the same injection volume and the same wavelength were used. The chromatograms are displayed in Figs. 7 and 8.

The next sample was GHRF. We observed differences on the chromatograms. However, this sample was unique in our selection of samples because of its very low protein concentration.

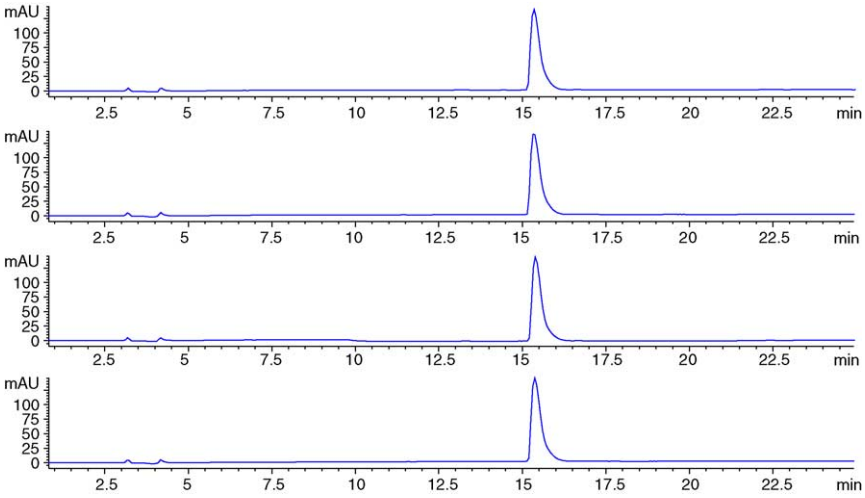

Fig. 7. RPLC chromatograms of insulin.

The chromatographic profile of the three samples is in general very similar, but some differences are apparent. A close investigation of the peaks reveals slight differences in the 11–13 min range. Also the peak area of the main peak around 15 min is significantly different for each sample. The discrepancies could be explained by the fact that we worked at a very low concentration at 0.03 mg/mL, the lowest sample concentration of all samples in this project. It is known that at a low concentration of peptides and proteins, adsorptions and or degradations could occur. It is important to note that at such a low concentration all peptide and protein pharmaceuticals could have similar problems. Appropriate formulation of peptides and proteins at low concentration could prevent adsorption related problems. The observed phenomena are interesting and should be further studied.

The RPLC profiles, of overlapping chromatograms of four somatostatin samples, show no extra peaks and indicate that no artifact was generated during needle free injections. We concluded that somatostatin seems to be unaffected by the delivery.

TRH was the smallest in the series of the studied model peptides with a molecular weight of 362 D. TRH eluted very early close to the system peak of our standard RPLC separation, which was designed for protein and not for small peptide separations and starts at 5% B solvent. Consequently the small TRH does not retain well on the column. However, comparing the chromatograms of TRH to the water blank, we can identify the sample related peaks, which apparently provide a very reproducible profile. Without further confirmation we used those peaks for the reproducibility studies. Good reproducibility and no extra peaks indicate stability of TRH during needle free injection.

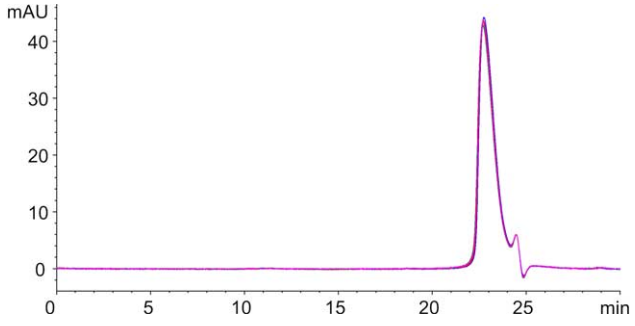

Fig. 8. SEC chromatograms of insulin. Overlay of four chromatograms, two needle free injection samples and the two standards.

The SEC analysis of these samples showed irregular elution behavior, such as unexpected multiple peaks and long irregular retention on the size-exclusion column. Consequently, we could not evaluate the SEC results.

9. Proteolytic enzymes

The proteolytic enzymes were represented by papain. The RPLC chromatograms of papain are shown in Fig. 9 and only one major peak can be observed. The RSD of the papain peak areas is over 5% and the samples are slightly different. At the beginning of the chromatograms where the small or hydrophilic compounds are eluting, we can see a few peaks, which are different on the different chromatograms. Another minor difference between the chromatograms is a little bump on the front of the main peak. The nature of these changes could be studied later but at this stage they are not considered significant. The peak area RSD is however slightly over the arbitrary limit. Since papain is a protease the RSD change could be the result of the timing of the measurement or self-degradation. As we observed earlier, the use of proteolytic enzymes as model proteins showed some changes. Whether those changes are due to the enzymes cannibalism alone or a combined effect of a conformational change during the pressure treatment followed by the proteolytic effect should be the subject of further studies.

As we mentioned earlier, the analysis of the proteolytic enzymes could be complicated. Due to the cannibalistic nature of the enzymes the chromatograms in some cases are

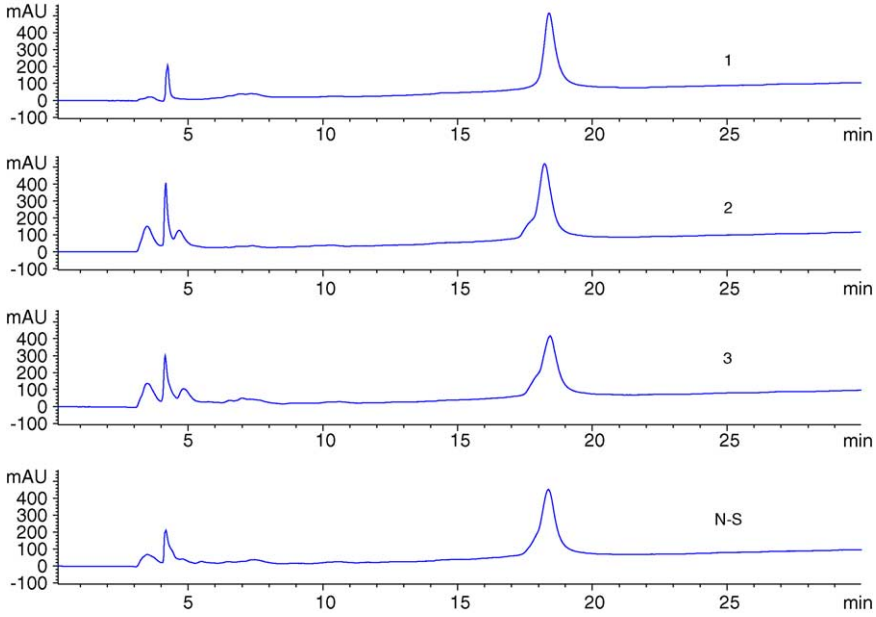

Fig. 9. RPLC of papain, 1–3 are the needle free injected samples and N–S is the chromatogram of the needle–syringe control.

too complex for meaningful evaluation. It is interesting that the general peak profiles are very similar within a series of single protein samples, which indicates that it is not likely that chemical changes are occurring. The trend is that the mass balance changes from experiment to experiment. The origins of those changes are unknown at this stage and more experimental work is needed to clarify this issue.

10. Conclusions

A total of 18 proteins were processed by a specific needle free injection device in order to acquire information on the possible effects of needle free injection on peptides and proteins. The overall goal of the study was to establish a baseline for the evaluation of needle free injection devices and provide experimental methods and data for further comparisons. We purposely used well-known and commercially available proteins to eliminate any possible questions regarding intimate and confidential details of biopharmaceutical products. The selected proteins are readily available and can be used for comparative evaluation by any scientist who works on the feasibility of using needle free devices for a company that is considering the implementation of this delivery option. The documented data could also serve as a reference data set for the evaluation of various types of needle free injection devices. The number of proteins, which were systematically studied under identical needle free injection conditions, provided some interesting information. The evaluations of the processed samples were performed by two standard HPLC methods, one a RPLC and the other an SEC method. Both methods are used regularly in our laboratory and the conditions selected are appropriate for general analysis. We did not develop customized conditions for each protein as part of our concept to use standard proteins and conditions for comparative purposes. In a couple of cases the standardized elution conditions were not appropriate for the particular samples and the analysis resulted in chromatograms, which were not useful for the comparisons. The RPLC method was selected to study potential peptide and protein degradation and the SEC to resolve the question of any potential injection induced aggregations.

The model proteins of this study could be divided into six different groups.

(1) The BSA samples present known aggregating proteins and aggregates (BSA).
(2) Ribonuclease A is a deglycosylated form of ribonuclease B, and they were selected to see the stability of glycosylated proteins during pressure injections. Similarly chymotrypsin and PEGylated chymotrypsin were intended to represent the family of PEGylated proteins.
(3) Cytochrome *c*, myoglobin and hemoglobin are proteins with prosthetic groups. They were selected to see the stability of heme containing proteins.
(4) Thyrotropin releasing hormone, growth hormone releasing factor, somatostatin and insulin are small peptides; they represented the family of peptide factors and hormones, which might be of therapeutic interest.
(5) Chymotrypsin, trypsin and papain represented the family of proteolytic enzymes.
(6) Individual proteins, which were selected because of general interest or available literature references, were β-lactoglobulin, lactic dehydrogenase, lysozyme and IgG.

We believe that the above reported experimental results support a reasonable argument that the application of needle

free injection does not necessarily damage the proteins. The evaluation protocol and applied methodologies seem to be appropriate to evaluate the two most critical consequences of needle free injections. The reported results can be considered as part of a database to which the performance of other devices can be compared.

The procedures, injections, controls and analytical methods represent a system to follow the events and possible consequences of needle free injection on peptides and proteins. The methods reported here are also applicable for comparisons and for the evaluation of future devices, new designs and modifications.

At this point the generated data strongly indicate that needle free injection device we used in these experiments does not degrade peptides and proteins and does not induce their aggregation either. The reproducibility of the peak areas of the samples, standards and processed samples are outstanding in most cases. Some of the proteins exhibited mass balance deficiencies. It seems that at low protein concentration some of the proteins have the tendency to stick to the injection system, a phenomena well known to protein formulators and chromatographers.

It is important to note that the above described evaluation system is recommended for the evaluation of all new biopharmaceuticals which could be candidates for needle free delivery. The fact that the selected samples seem to be unaffected does not necessary mean that some proteins could not behave anomalously.

In general, the questions raised and situations recall the early development period of HPLC when we also had to answer the same questions and observed similar variations from protein to protein.

We sincerely hope that this work will assist in the reconsideration of the "phobias" diagnosed and named by Csaba Horváth, the "father of HPLC" more than 20 years ago, as they reappear today to hinder the acceptance of needle free injection. Any barophobia, siderophobia or lithophobia surrounding the use of needle free injection can be dispelled by scientific methods, including HPLC, which was pioneered by Professor Horváth.

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