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Journal of Chromatography B, 821 (2005) 45–52
JOURNAL OF CHROMATOGRAPHY B
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HPLC and tandem detection to monitor conformational properties of biopharmaceuticals

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Received 7 October 2004; accepted 9 April 2005
Available online 17 May 2005

Abstract

High-performance liquid chromatography (HPLC) with UV, circular dichroism (CD) and intrinsic fluorescence detection was applied to monitor conformational properties of recombinant human interferon α2b when performing size exclusion chromatography (SEC) and reversed-phase HPLC (RP-HPLC). In this way native conditions during SEC and structural changes of the protein during RP-HPLC were demonstrated. These results were confirmed by stand-alone fluorescence and CD measurements. With respect to HPLC tandem detection, the fluorescence detector compared favourably to the UV and CD detector regarding linearity, sensitivity and selectivity. SEC combined with intrinsic fluorescence scanning detection permits conformational analysis of small amounts of aggregates in the presence of excess native monomeric protein. In conclusion, HPLC with on-line UV and intrinsic fluorescence detection provides a promising concept for analysing the amount and conformational properties of a biopharmaceutical and its impurities.

Keywords: (Reversed-phase) HPLC; Biopharmaceuticals; Protein conformation; Intrinsic fluorescence; Circular dichroism; Tandem-detection; Interferon-alfa; Human growth hormone; Anti tetanus immunoglobulin; Human serum albumin

1. Introduction

High-performance liquid chromatography (HPLC) is a widely used technique for investigating the content and purity of medicinal products including biopharmaceutical proteins [1–3]. A major difference between protein-based and small-molecule pharmaceuticals is that the bioactivity of proteins not only relies on the primary molecular structure but also on a higher order structure, i.e., molecular conformation. This presents difficulties in the development of content assays for biological molecules based on HPLC. An ideal content assay should provide information on the correct conformation of the protein as well. Although suitable tools are available for characterising primary structural aspects of proteins (e.g., sequencing, mass spectrometry, HPLC, electrophoresis), these tools as such cannot reveal whether the protein is in the correct, folded structure. However, higher order structural aspects can be revealed by spectroscopic technologies such as nuclear magnetic resonance, Fourier transform infrared, circular dichroism (CD) and intrinsic fluorescence or to some extent by assays based on biological responses.

In combination with UV-detection HPLC is a separation method that provides a powerful means for characterising the homogeneity of protein samples. Reversed-phase HPLC (RP-HPLC) is suited for this purpose because of its high resolution. RP-HPLC separates proteins based on subtle differences in hydrophobicity. Small to mid sized proteins (up to 25 kDa) which differ by a single amino acid residue can often be separated by this method. Therefore, RP-HPLC is

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doi:10.1016/j.jchromb.2005.04.005

often applied for quantification of pharmaceutical proteins and for the analysis of closely related protein variants or degradation products (e.g., oxidised, deamidated) [1–3]. In this way, RP-HPLC plays an important role in research & development, production and quality control of biopharmaceuticals. RP-HPLC requires the use of an organic modifier (usually acetonitrile (ACN)) and ion-pairing agent (usually trifluoroacetic acid (TFA)) to achieve optimal separation conditions [4]. Unfortunately, typical RP-HPLC conditions could affect the conformation of the protein [5] so the detector response does not necessarily represent the amount of bioactive compound. Another type of HPLC commonly applied for analysing biopharmaceuticals is size exclusion chromatography (SEC). In this case proteins are separated on size and shape. SEC may be used for the quantification of proteins but it is normally applied to determine the native size of the protein and to reveal possible multimers and aggregates [6–8]. In contrast to RP-HPLC, SEC is performed under separation conditions which are expected not to affect the higher order structure of the protein.

In the present study we envisaged that the combination of structural specific separation and conformation selective detection offers possibilities for improved assay designs. In the first series of experiments, we investigated the possibilities to detect conformational properties of recombinant interferon-alfa 2b (IFN-α2b) on line during SEC and RP-HPLC. A scanning fluorescence and a CD detector were coupled to an HPLC system with an UV detector that monitors exclusively the amount of protein. Far-UV CD spectroscopy enables us to study the secondary structure elements in the proteins while intrinsic fluorescence spectroscopy provides complementary information on changes in the local environment of the aromatic side chains (tertiary structure). The on-line spectroscopic results were compared with stand-alone fluorescence and CD measurements to reveal the effect of RP-HPLC mobile phase compositions on the IFN-α2b conformation. Next to this, the performances of the fluorescence and CD detector were compared to those of the UV detector. In the second series of experiments HPLC in combination with tandem detection was applied to analyse several biopharmaceutical preparations on protein aggregates. Eventually, this investigation demonstrates the value of tandem detection to evaluate the effects of chromatographic conditions on the conformation of a biopharmaceutical. This study may contribute to the development of straightforward and fast methods to monitor the content as well as conformational properties of the active compound and impurities in biopharmaceutical products.

2. Experimental

2.1. Reagents

The IFN-α2b standard solution (PhEur, CRS, batch 3) was supplied by the European Directorate for the Quality of Medicines (EDQM, Strasbourg, France). The supplied solutions contained approximately 7.19 mg/ml IFN-α2b. The recombinant *N*-methionyl human growth hormone (Met-hGH, order 6907) preparation 'Somatrem' (sampled by health authorities from an illegal distribution channel) contained approximately 4 IU (1.3 mg) lyophilised Met-hGH per vial. The anti tetanus immunoglobulin (TIG) preparation 'Tetaquin' (Sanquin, The Netherlands) contained 10–18% protein, predominantly (>90%) TIG. The human serum albumin (HSA) preparation 'Cealb' (Sanquin, The Netherlands) contained 20% protein predominantly (>95%) albumin. Phosphate buffered saline, pH 7.2 (PBS), consisted of 8 mM Na_2HPO_4, 2 mM NaH_2PO_4 and 154 mM NaCl. Solvents for chromatography were HPLC grade and salts were analytical grade chemicals.

2.2. Sample preparation

2.2.1. Stand-alone measurements

Standard solutions of IFN-α2b were diluted with ACN/water mixtures or ACN/water mixtures in presence of 0.1% TFA (v/v) to protein concentrations of 50 μg/ml (fluorescence measurements) and 250 μg/ml (CD measurements). The prepared protein solutions contained 10, 20, 30, 40, 50 and 60% (v/v) of organic solvent, they were kept at room temperature for 1 h before spectroscopic measurements.

2.2.2. HPLC measurements

Standard solutions of IFN-α2b were diluted with PBS to a concentration of 100 μg/ml before being applied on RP-HPLC or SEC.

2.2.3. Protein aggregates

Soluble aggregates of IFN-α2b were prepared by incubating the protein in 50 mM potassium phosphate buffer (100 μg/ml), pH 8.5, at 40 °C for 7 weeks [9]. For Met-hGH, soluble protein aggregates were prepared by vortexing protein solutions of 1 mg/ml for 3 min. TIG (10 mg/ml) and HSA (10 mg/ml) samples contained detectable levels of soluble aggregates without pretreatment.

All samples and solutions were filtered before use over a 0.45 μm filter.

2.3. Fluorescence spectroscopy

Fluorescence spectra were obtained with an LS-50B spectrofluorometer (Perkin Elmer) at 25 °C in a 1-cm quartz cuvette (Hellma GmbH, Müllheim, Baden, Germany) with a protein concentration of 50 μg/ml. An excitation wavelength of 295 nm was used, with a band pass of 2.5 nm for the excitation monochromator and 5.0 nm for the emission monochromator. When excited at 295 nm, the maximum wavelength of the fluorescence emission spectrum is indicative of the degree of solvent exposure of the side chains of the tryptophan (Trp) residues. Data were recorded at 1 nm intervals over the range 300–500 nm with a scanning speed of 300 nm/min and data were smoothed after acquisition (five passes). Fluorescence

spectra were corrected for background signal contributed by buffer.

2.4. Circular dichroism spectroscopy

Far-UV CD spectra were recorded on a dual-beam DSM 1000 CD spectrophotometer (On-Line Instrument Systems, Bogart, GA) at 25 °C. The subtractive double-grating monochromator was equipped with a fixed disk, holographic gratings (2400 lines/mm, blaze wavelength 230 nm), and 1.24-mm slits. The protein concentration used was 250 μg/ml. For far-UV CD spectra (250–200 nm), samples were measured in a 0.05 cm path length 200 μl quartz cuvette (Hellma GmbH, Müllheim, Baden, Germany). All CD spectra resulted from averaging six repeated scans (step resolution 1 nm, 1 s each step). Subsequently, spectra were background-corrected and smoothed. The measured signals were converted to molar absorbance difference ($\Delta\varepsilon$ in $M^{-1}\,cm^{-1}$), based on a mean residual weight of 110 Da per amino acid residue.

2.5. HPLC

2.5.1. Instrumentation

HPLC experiments were performed on two different systems. The first one included a Jasco AS-950 Intelligent sampler, Jasco PU-980 Intelligent HPLC pump, Jasco LG-980-02 ternary gradient unit, Alltech on-line degassing system, Spectra Physics SP8790 column oven, Jasco MD-2010 Plus multiwavelength detector, Jasco CD-1595 detector. The HPLC system was controlled by Borwin software version 1.5. UV detection was performed at 214 nm, CD detection at 220 nm (gain 10×). The CD 220 nm signal peak area was related to the UV 214 nm signal peak area in order to normalise for protein concentration.

The second HPLC system was an Agilent 1100 system including a G1379A micro vacuum degasser, G1312A binary pump, G1329A auto-sampler, G1330B auto-sampler thermostat, G1316A thermostatted column compartment, G1315B diode array detector and G1321A fluorescence scanning detector. This Agilent HPLC system was operated by ChemStation software. UV detection was performed at 214 nm, fluorescence emission detection at 338 nm (SEC) or 342 nm (RP-HPLC) with an excitation wavelength of 295 nm. The fluorescence spectra were measured at 1 nm intervals over the range 320–400 nm with a scanning speed of 80 nm/s (PMT-gain 11).

2.5.2. Size exclusion chromatography

IFN-α2b (0.5, 1.5, 3.0, 6.0, 8.0 and 10 μg) was applied to a TSKgel G3000SW$_{XL}$ column (300 mm × 7.8 mm, 5 μm, 250 Å), equilibrated with PBS. The elution was performed with the equilibration buffer at a flow rate of 0.8 ml/min and 25 °C.

IFN-α2b and Met-hGH containing soluble protein aggregates were applied to a TSKGel G2000SW$_{XL}$ column (300 mm × 7.8 mm, 5 μm, 125 Å). The elution was performed with PBS at a flow rate of 0.8 ml/min and 25 °C. TIG and HSA containing soluble aggregates were applied to a TSKGel G3000SW$_{XL}$ column (600 mm × 7.8 mm, 5 μm, 250 Å) and eluted as described in the PhEur 2002 [10,11].

2.5.3. Reversed-phase HPLC

IFN-α2b (0.5, 1.5, 3.0, 6.0, 8.0 and 10 μg) was applied to a Vydac 218TP54 C_{18} column (250 mm × 4.6 mm, 5 μm, 300 Å), equilibrated with 38% ACN (v/v) and 0.1% TFA (v/v). RP-HPLC was performed at 25 °C with a gradient of ACN from 38 to 70% in presence of 0.1% TFA at 1 ml/min as described by Buchheit et al. [2].

3. Results

3.1. Effect of RP-HPLC conditions on IFN-α2b

To validate the use of fluorescence and CD detection for the purpose of monitoring conformational changes, stand-alone experiments were performed. In addition, the effect of common RP-HPLC mobile phase compositions on the molecular conformation (secondary and tertiary structure) of IFN-α2b was investigated. Therefore, intrinsic fluorescence and far-UV CD spectra of IFN-α2b in presence of ACN or ACN and 0.1% TFA were measured. The concentrations of the organic solvent were 0, 10, 20, 30, 40, 50 and 60% (v/v).

3.1.1. Stand-alone fluorescence measurements

The intrinsic Trp fluorescence spectrum of IFN-α2b in PBS and in absence of organic solvent (native conditions) revealed that the fluorescence emission maximum (F_{max}) was 338 nm (Fig. 1). This indicates that the Trp residues were partially buried in the hydrophobic core of the protein. The maximum remained approximately the same for the protein up to ACN concentrations of 20%. At higher concentrations of ACN, F_{max} increased to 342 nm suggesting a change to a more polar environment for the emitting Trp residues in IFN-α2b. This red-shift of F_{max} also occurred as a result of incubation of the protein with increasing concentrations of ACN in presence of 0.1% TFA (from 338 to 342.5 nm). It was noticed that the fluorescence intensity increased as the concentration of the organic solvent increased (not shown).

Since Trp fluorescence is highly sensitive to solvent conditions, the effect of the RP-HPLC mobile phases on Trp fluorescence was examined by carrying out studies with *N*-acetyltryptophanamide (NATA) under these conditions (data not shown). NATA is a low molecular weight compound containing the Trp structure. Fluorescence spectra of NATA in presence of ACN or ACN and 0.1% TFA showed a similar increase of fluorescence intensity as was observed for IFN-α2b when the concentrations of the organic modifiers were increased. Furthermore, a decrease of F_{max} from 357 to approximately 350 nm was measured for NATA as

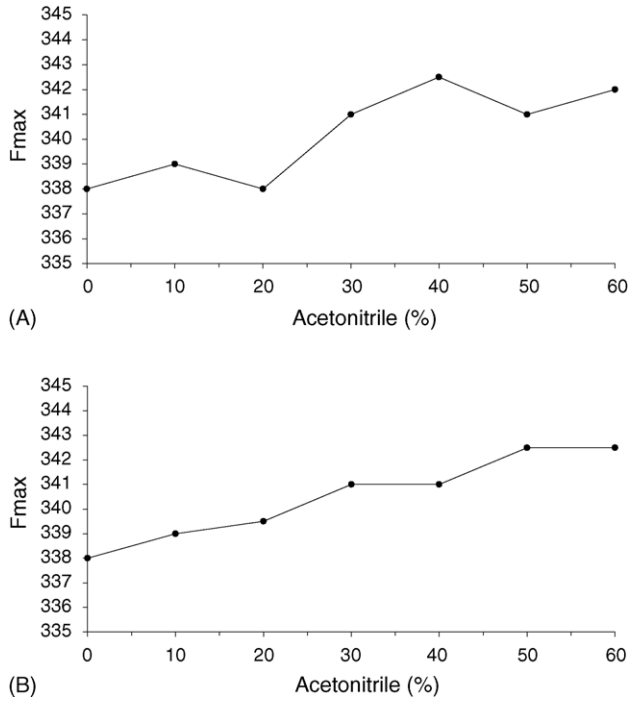

Fig. 1. Effect of concentration (A) acetonitrile and (B) acetonitrile in presence of 0.1% TFA on the intrinsic fluorescence emission maximum of IFN-α2b.

a result of the increasing hydrophobicity of the solvents. This was in contrast to what was observed for IFN-α2b which means that the increase of F_{max} observed for the protein in presence of increasing amounts of RP-HPLC organic modifier indeed reflects modifications of the protein structure.

3.1.2. Stand-alone CD measurements

Far-UV CD-spectra of IFN-α2b in presence of 0–60% ACN are shown in Fig. 2A. These spectra were taken to obtain information about the secondary structure of the protein. In absence of organic modifier (native conditions) the CD spectrum of IFN-α2b showed a $\Delta\varepsilon$-minimum around 211 and 222 nm. The shape of the spectrum indicated that IFN-α2b mainly consists of α-helices. This is in agreement with the 3D-structure of IFN-α2b published in protein databases. The spectra of IFN-α2b in presence of 30% ACN and higher concentrations exhibited a decrease of the overall broad negative $\Delta\varepsilon$-intensity indicating partial denaturation of the protein. In presence of 60% ACN it appeared that all α-helices were lost and anti-parallel β-sheets remained [12].

The far-UV CD spectra of IFN-α2b in presence of 10% ACN and 0.1% TFA revealed a shift of the $\Delta\varepsilon$-minimum from 222 to 224 nm and a broadening of the negative $\Delta\varepsilon$-intensity (Fig. 2B). When higher concentrations of ACN in presence of 0.1% TFA were applied the negative $\Delta\varepsilon$-intensity increased at 211 nm and to a smaller extent also at 224 nm. These increases suggest subtle changes in secondary structure distinct from those in the absence of TFA.

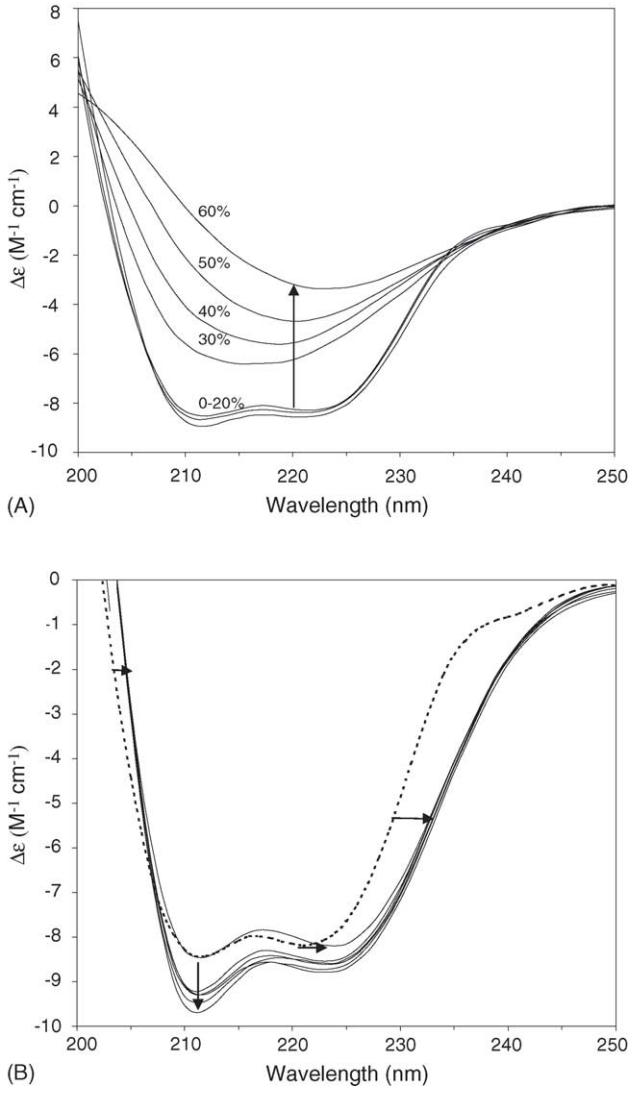

Fig. 2. Far-UV CD spectra of IFN-α2b (A) in presence of 0, 10, 20, 30, 40, 50 and 60% acetonitrile and (B) in presence of 0 (dotted line), 10, 20, 30, 40, 50 and 60% acetonitrile and 0.1% TFA.

3.1.3. HPLC in combination with tandem-detection

0.5–10 μg IFN-α2b applied to SEC eluted isocratically with PBS around 14 min (not shown). IFN-α2b applied to RP-HPLC eluted between 30 and 34 min (depending on the HPLC system used) at approximately 50% ACN and 0.1% TFA (Fig. 3). As expected, for both SEC and RP-HPLC, a positive UV and intrinsic fluorescence emission signal and negative CD signal were obtained (Fig. 3). The corresponding peak areas were proportional to the amount of protein applied (Table 1).

By using a scanning fluorescence detector it was not only possible to monitor one or more fluorescence emission wavelengths but also to measure fluorescence spectra on-line. In this way the F_{max} values could be revealed. The average F_{max} over the range 0.5–10 μg IFN-α2b obtained with SEC and RP-HPLC was determined to 338.8 and 344.2 nm, respectively (Table 1). Any changes in Trp fluorescence reflect an

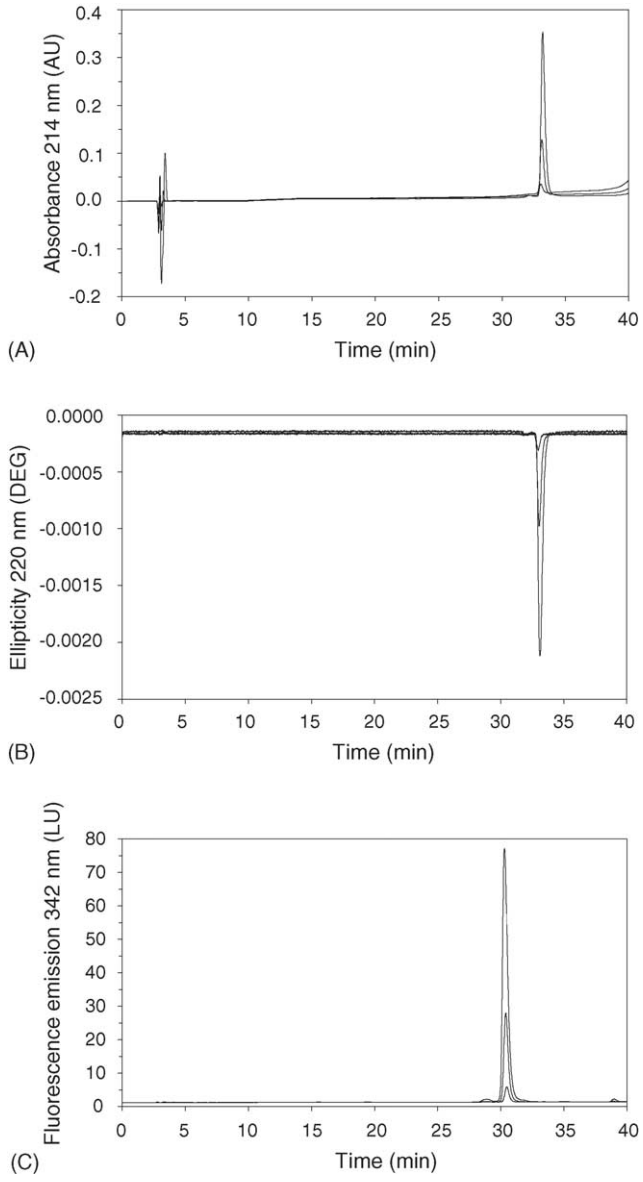

Fig. 3. HPLC UV (A), CD (B) and intrinsic fluorescence (C) profile of 0.5, 3.0 and 10.0 μg IFN-α2b applied to RP-HPLC. (A) and (B) were obtained by the Jasco HPLC system, (C) was obtained by the Agilent HPLC system.

alteration in the environment of the residue. This means that the tertiary protein structure during SEC differs from that during RP-HPLC. The average CD_{220}/UV_{214} peak area ratio over the range 0.5–10 μg IFN-α2b applied to SEC appeared to be similar to the ratio obtained with RP-HPLC, taking into account that the standard deviation was relatively high (Table 1).

3.2. Performance of HPLC detectors

The UV, CD and fluorescence emission signals were linear over the range 0.5–10 μg IFN-α2b (Table 1). This means that the corresponding detectors are applicable for analysing common biopharmaceutical products. However, within the concentration range investigated the CD signal appeared to show a poorer linearity compared to that of the UV and fluorescence emission signal. Furthermore, the UV signal measured by the Agilent PDA detector showed a better linearity and sensitivity than that of the UV signal measured by the Jasco PDA detector. The LOD of the UV, CD and intrinsic fluorescence detection was estimated to be 5–10, 100 and 1 ng of IFN-α2b, respectively (Table 1). This indicated that CD detection is less sensitive than UV and fluorescence detection. The overview presented in Table 1 shows a clear relationship between the sensitivity of a detector (LOD) and the linearity (R^2).

CD detection is more selective than UV detection because only chiral compounds are detected. This also implies that less matrix interference will occur. Intrinsic fluorescence detection was more sensitive than UV detection. Moreover it has a higher selectivity than UV detection as only specific fluorescent compounds are detected. This selectivity is dependent on the selected excitation wavelength.

3.3. Protein aggregates

The intrinsic fluorescence behaviour of various protein aggregates has been investigated and compared to that of the corresponding native protein (Table 2). Therefore, IFN-α2b, Met-hGH, TIG and HSA samples containing significant amounts of aggregates have been applied to SEC combined with a fluorescence scanning detector. In all chromatograms the aggregates eluted in peaks well separated before the main compounds. The aggregate fluorescence spectra of IFN-α2b, Met-hGH and TIG revealed a shift of the F_{max}. In case of IFN-α2b (Fig. 4) and TIG, the F_{max} of the aggregates was higher compared to that of the corresponding native protein. This indicated a change to a more polar environment for the emitting Trp residues in the aggregates. IFN-α2b dimers (retention at 11 min), which elute between aggregate (7.5 min) and monomer (12 min) peaks, revealed a similar F_{max} as was found for the monomer. For Met-hGH the F_{max} of the aggregates was lower indicating a more hydrophobic surrounding for the Trp residues. For HSA, the native protein and its aggregates showed similar F_{max} values.

4. Discussion

Physicochemical methods have been successfully applied to determine the structural identity, purity, integrity and stability of various biopharmaceuticals [3,13–18]. Our aim was to study the possibilities of HPLC combined with UV, CD and intrinsic fluorescence (tandem) detection for monitoring conformational properties of several biopharmaceuticals. Whereas CD detection monitors the secondary protein structure, fluorescence detection monitors the tertiary protein structure. We used RP-HPLC and SEC to test this tandem system and to reveal the effects of chromatographic conditions on the protein conformation of IFN-α2b. Stand-alone fluo-

Table 1
Overview of the various results obtained by UV, CD and intrinsic fluorescence detection when 0.5–10 μg IFN-α2b was applied to SEC and RP-HPLC; regression statistics, CD_{220}/UV_{214} ratio, F_{max} and LOD

	Equation	R^2	CD_{220}/UV_{214} ratio	F_{max} (nm)	LOD (ng)
SEC					
Jasco					
UV (214 nm)	$y = 999211x + 154385$	0.9949			10
CD (220 nm)	$y = 6747x + 2363$	0.9912	0.0072 ± 0.0004		100
Agilent					
UV (214 nm)	$y = 828.9x - 164.9$	0.9997			5
Fluor. (338 nm)	$y = 166.1x - 20.7$	0.9999		338.8 ± 0.8	1
RP-HPLC					
Jasco					
UV (214 nm)	$y = 818078x + 27241$	0.9973			10
CD (220 nm)	$y = 5193x + 1495$	0.9886	0.0070 ± 0.0006		100
Agilent					
UV (214 nm)	$y = 1117.6x - 27.9$	1			5
Fluor. (342 nm)	$y = 222.0x + 4.6$	0.9999		344.2 ± 1.2	1

rescence and CD measurements were done to evaluate the results obtained by HPLC detection.

Intrinsic fluorescence and far-UV CD spectra of IFN-α2b in presence of commonly used RP-HPLC mobile phases showed that the tertiary and secondary structures of the protein were affected (Figs. 1 and 2). Whereas hardly any structural alterations were observed for the protein in 20% and lower concentrations of ACN, altering of tertiary structure and loosening of secondary structure were demonstrated in 30–60% ACN (Figs. 1A and 2A). Loosening of secondary structure indicates denaturation of the protein. When the protein was incubated with 10–60% ACN in presence of 0.1% TFA the tertiary structure was altered but IFN-α2b was not denatured (Figs. 1B and 2B). This means that TFA prevents protein denaturation by high concentrations of ACN. TFA is an ion-pairing reagent that sets the low eluent pH and interacts with the polypeptide to enhance the HPLC separation. It is also known that TFA has denaturing properties [19]. For example, it was demonstrated that TFA (concentrations around 0.1%) induces extensive unfolding of cytochrome *c* characterised by a significant breakdown of the secondary and tertiary structure of the protein [20]. Such an effect was not observed in the present study for IFN-α2b in presence of ACN and TFA. Yet the secondary as well as the tertiary structure of the protein were altered (Figs. 1B and 2B); a relatively more β-structure was obtained. This may explain to some extent the benefits of TFA for resolution of IFN-α2b in RP-HPLC as was observed by Buchheit et al. [2].

Table 2
The F_{max} of various native biopharmaceuticals and their aggregates obtained via SEC with intrinsic fluorescence scanning detection

Biopharmaceuticals	F_{max} native (nm)	F_{max} aggregates (nm)
IFN-α2b	339.1 ± 0.3	342.4 ± 0.5
Met-hGH	339.1 ± 0.3	337.3 ± 0.5
TIG	338.5 ± 0.7	340.9 ± 0.7
HSA	342.5 ± 1.3	342.8 ± 0.4

HPLC combined with CD detection was applied to find out if this method is suitable to monitor secondary structural changes of IFN-α2b. Therefore, the CD_{220}/UV_{214} peak area ratio was determined for the protein when applied to SEC and RP-HPLC (Table 1). In view of the stand-alone far-UV CD measurements one would expect a somewhat higher CD_{220}/UV_{214} peak area ratio with RP-HPLC than SEC, as the negative $\Delta\varepsilon$-intensity at 220 nm was slightly higher in 50% ACN and 0.1% TFA (Fig. 2B). However, the CD_{220}/UV_{214} ratio obtained by RP-HPLC on-line appeared to be somewhat lower (Table 1). Due to the relatively poor performance of the CD detector at this low protein concentration level it is not clear whether these $\Delta\varepsilon$-intensity differences are significant. It seems that at this point CD detection at 220 nm is only interesting in case of major secondary structural changes of the protein. For example, fully denatured proteins (no secondary structure) would result in very low CD_{220}/UV_{214} ratios. Therefore, the CD_{220}/UV_{214} peak area ratio measured by our HPLC system is not sensitive enough for the detection of minor secondary structural changes. The lower sensitivity of the CD detector is due to the fact that CD detection is based on absorption differences between right and left circularly polarised lights [21]. Although CD detection at several wavelengths would give more information about the structural changes of a protein on-line, the sensitivity would still be not sufficient for analysing low protein doses occurring in biopharmaceutical products. Next to this, the CD detector is only capable of monitoring one wavelength and from 220 nm. In view of the far-UV CD spectra it would be very informative to monitor also at lower wavelengths (Figs. 2–4). Eventually, a more sensitive and scanning CD-detector from 200 to 250 nm is needed to monitor the secondary structure of proteins on-line accurately.

With respect to HPLC in combination with intrinsic fluorescence scanning detection, the obtained fluorescence spectra were analysed to retrieve information from the tertiary protein structure of IFN-α2b during SEC and RP-HPLC.

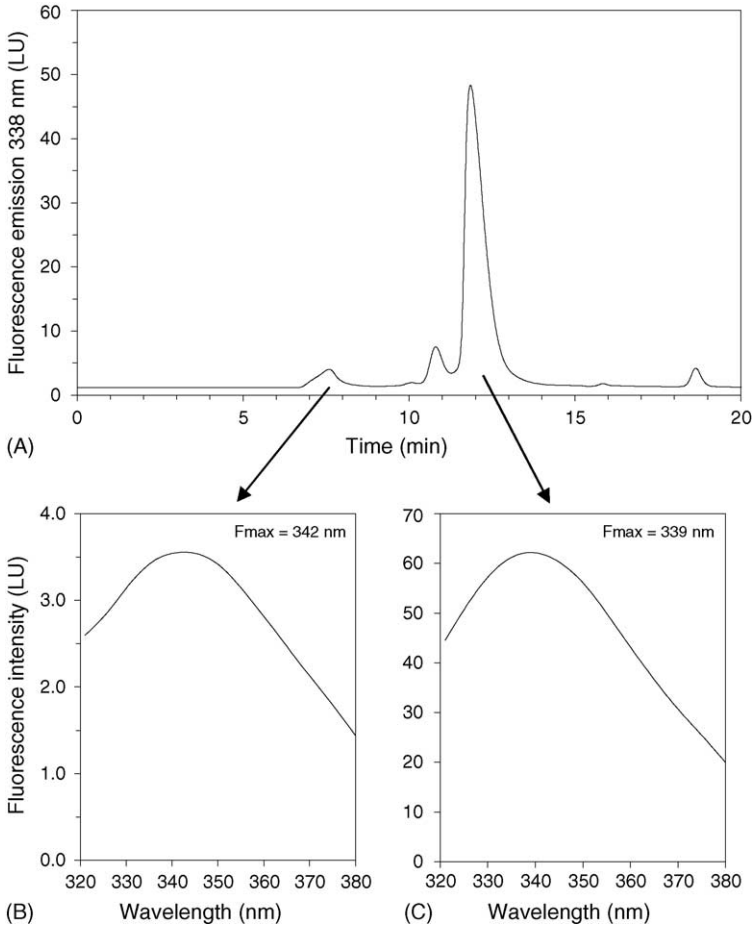

Fig. 4. HPLC intrinsic fluorescence profile of 10.0 μg IFN-α2b containing aggregates when applied to SEC (A) and the corresponding intrinsic fluorescence spectrum of IFN-α2b aggregates (B) and native IFN-α2b (C).

When 0.5–10 μg IFN-α2b was applied to SEC with PBS as mobile phase, the F_{max} was 338.8 ± 0.8 nm (Table 1). This F_{max} resembles the F_{max} (338 nm) obtained by stand-alone fluorescence measurements on IFN-α2b in PBS (Fig. 1) and it confirms the native conditions applied with SEC. The F_{max} of IFN-α2b applied to RP-HPLC was determined to be 344.2 ± 1.2 nm (Table 1). Taking into account that the protein eluted at approximately 50% ACN in presence of 0.1% TFA, the corresponding F_{max} obtained by stand-alone measurements was 342.5 nm (Fig. 1B). Additional experiments with the fluorescence detector in a stand-alone setting (data not shown) revealed that the F_{max} was 344 nm. This suggests slight differences between calibration of the spectrofluorometer and fluorescence scanning detector. In any case, these F_{max} values indicate that the tertiary protein structure of IFN-α2b was affected by the RP-HPLC conditions. Overall, the results obtained by HPLC in combination with fluorescence scanning detection correlate with the results obtained by the stand-alone measurements. Therefore, this HPLC set up is suitable to monitor structural changes of the protein on-line and reveals information about the folded state of the applied protein; native, structurally altered or denatured. In the latter case the F_{max} value would exceed 350 nm [13,15]. Next to the structural information obtained by HPLC-fluorescence, this detection mode showed other advantages compared to HPLC-UV. As shown by the LOD value in Table 1, intrinsic fluorescence detection appeared to be more sensitive than UV detection in the analysis of IFN-α2b. In general, the intrinsic fluorescence intensity is dependent on the amount and location of the Trp residues in the protein molecule. In case of IFN-α2b two Trp residues are present per molecule. If no Trp residue would be present in a protein molecule one can consider to excite at 280 nm. In this way the F_{max} of the fluorescence emission spectrum is indicative of the degree of solvent exposure of the side-chains of tyrosine [22]. Another practical advantage of HPLC-fluorescence above HPLC-UV is the higher selectivity of intrinsic fluorescence detection (see Fig. 3A and C). In general, matrix compounds such as pharmaceutical excipients and buffer components do not interfere in the intrinsic fluorescence chromatogram. Therefore, more simple chromatograms will be obtained and peaks can be easily attributed to be proteinaceous compounds or not.

HPLC combined with intrinsic fluorescence scanning detection has also been applied to reveal possible fluorescence differences on-line between a native protein and its aggregates (Fig. 4). In case of protein aggregation a shift of the

F_{max} to lower wavelengths was expected as relatively more Trp residues would be surrounded by other protein molecules resulting in a more hydrophobic environment. This was observed in the case of Met-hGH aggregates. Whereas the F_{max} of the aggregates was 337.3 nm, the F_{max} of the native protein was 339.1 nm (Table 2). Human growth hormone is known to be highly resistant to denaturation and to form easily associated states [23,24]. In contrast to Met-hGH, for IFN-α2b (Fig. 4) and TIG the F_{max} of the aggregates was higher than that of its native protein. This could indicate another mechanism of aggregation. It is known that aggregation not only results from interaction of protein molecules in the native state but also from the interaction of protein molecules in the unfolded state with another unfolded protein [25]. In case of protein unfolding the F_{max} will increase as the emitting Trp residues will be more exposed to the solvent (more polar environment). In case of HSA the F_{max} of native HSA equals that of its aggregates. This is probably due to the location and amount of Trp residues present in HSA and the relatively large size of the native molecule. HSA contains only a single Trp residue that is found to be deeply buried in the protein [26]. In this way the Trp environment is not easily affected by processes that occur on the more outer part of the molecule. Probably the HSA aggregates were formed via accumulation of native protein molecules as a shift of the F_{max} was probably observed in case the HSA molecules were first unfolded.

In conclusion, our investigation demonstrates the possibility to acquire information on conformational properties of HPLC-separated proteins on-line by tandem detection. This set up has been successfully applied to reveal conformational changes of IFN-α2b during RP-HPLC analysis and to discriminate conformational properties of aggregates from the corresponding native protein during SEC. In particular the use of intrinsic fluorescence scanning detection provides additional selectivity and sensitivity in protein HPLC. Such a detection system provides a promising concept to develop straightforward and fast methods for assaying the purity and content of native (i.e., biologically active) protein in biopharmaceutical preparations. In this study we presented an example of an RP-HPLC content assay which does not directly measure the protein in its active form. Milder chromatographic conditions such as those applied during hydrophobic interaction or ion-exchange chromatography should be developed in order to separate proteins in their native conformational state. The use of tandem-spectroscopic detection systems facilitates the evaluation of these new methods which ultimately may replace biological assays.

Acknowledgements

The authors are grateful to the Unit Research and Development of the Netherlands Vaccine Institute for their assistance during the fluorescence measurements and to Suzanne Hermeling and Nataša Jovanović (Department of Pharmaceutics, Utrecht University) for their assistance with the CD measurements. The authors acknowledge Michel Girard (Health Canada, Centre for Biologics Research) for critically reading the manuscript and fruitful discussions.

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