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ANALYSIS OF DIFFERENT FORMS OF RECOMBINANT HUMAN LEUKOCYTE INTERFERONS AND SYNTHETIC FRAGMENTS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Analytical high-performance liquid chromatography (HPLC) conditions are reported for the evaluation of recombinant human interferon monomers: recombinant human leukocyte interferon A (rIFN-αA), rIFN-αD, and the hybrid rIFN-$\alpha A_{1\text{-}62}/D_{64\text{-}166}$. The two monomeric forms of rIFN-αA (slow-migrating monomer and fast-migrating monomer) were also resolved by HPLC. Conditions are reported for the HPLC separation of oligomers (dimers and trimers) of rIFN-αA. The synthesis and analytical HPLC of the carboxy-terminus fragment, corresponding to IFN-αD (140–166), and a series of analogues comprising the IFN-αA (105–125) region is reported. The syntheses were accomplished by the solid-phase peptide synthesis procedure and the products were purified by preparative HPLC.

INTRODUCTION

The cloning and expression of the recombinant human leukocyte interferon A (rIFN-αA) gene in *Escherichia coli*[1] and subsequent purification by the use of immobilized monoclonal antibodies[2] has led to the scaled-up production[3] of rIFN-αA for human clinical trials[4]. Analysis of the resultant rIFN-αA by non-reducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis[5] (SDS-PAGE) revealed the presence of higher oligomers (dimers, trimers, tetramers, etc.) and two monomeric forms which have been termed "slow"- and "fast"-migrating monomers (SMM and FMM, respectively) to designate their relative mobilities after SDS-PAGE.

Although disulfide linkages[6] in rIFN-αA were determined to reside between Cys^1–Cys^{98} and Cys^{29}–Cys^{138}, it has been suggested that only the Cys^{29}–Cys^{138} disulfide bridge is required for biological activity[7]. When Cys^1 and Cys^{98} were replaced by using *in vitro* DNA mutagenesis[8], only the SMM was found. This suggested the involvement of Cys^1 and Cys^{98} in intermolecular disulfide-bonded oligomers. It can be reasoned that the FMM could represent a monomer with both disulfide bonds intact, whereas the SMM represents a partially reduced form with only the Cys^{29}–Cys^{138} bond intact. This mixture of monomers has recently been separated by using a modification of the metal chelate chromatographic method of Porath[9] and Houchuli[10].

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Although the components of rIFN-αA can be separated by SDS-PAGE, high-performance liquid chromatography (HPLC) is an alternative method which may be advantageous for routine analysis of multiple samples. Preparative HPLC of human leukocyte interferon has been successfully carried out by reversed phase on LiChrosorb RP-8[11,12]. We have examined a number of analytical HPLC procedures (for recent reviews see refs. 13 and 14) for possible use in the rapid analysis of rIFN-αA (and oligomers), the related interferon species rIFN-αD, and the hybrid rIFN-$\alpha A_{1-62}/D_{64-166}$ (Bgl II)[15] [rIFN-αA/D] (Fig. 1).

Our interest in defining the localization of the epitopes for the monoclonal antibodies LI-1 and LI-8 on the IFN-αA molecule[16] necessitated the synthesis of a series of IFN-αA fragments and analogues. To this end, IFN-αA (105–125) and IFN-αA [Val[113]] (105–125) were prepared and evaluated for their ability to compete with rIFN-αA for binding to LI-1 and LI-8[16]. In addition, the corresponding 3,4-dehydroprolyl residue (Δ^3 Pro) was introduced at position 109 for subsequent radiolabeling[17]. Moreover, a novel fragment, comprising IFN-αD (140–166) was prepared for production of non-neutralizing antibodies[18]. The synthesis and analytical HPLC of these peptides are also reported in this paper.

EXPERIMENTAL

Materials

All solvents used were purchased from Burdick & Jackson (distilled-in-glass grade). Dimethylformamide (DMF) was distilled from ninhydrin at reduced pressure and stored over Molecular Sieve (4Å) (Linde, Morristown, NJ, U.S.A.). Trifluoroacetic acid (TFA) and diisopropylethylamine (DIEA) (Chemical Dynamics, South Plainfield, NJ, U.S.A.) were of Sequalog-grade purity. Hydroxybenzotriazole and dicyclohexylcarbodiimide (DCC) (Sequenal-grade purity) were purchased from Pierce, Rockford, IL, U.S.A. Distilled water was further purified in a Hydro Model DC 1-18 system (Hydro Inc., Paramus, NJ, U.S.A.) consisting of a 0.5-μm filter cartridge, organic adsorption unit, and mixed-bed ion-exchange unit to give water with a specific resistance in excess of 18 MΩ/cm^3. Perchloric acid (70%) was of reagent grade purity (Fisher Scientific, Fairlawn, NJ, U.S.A.) and used to prepare a 0.1% aqueous solution, which was adjusted to pH 2.5 with 1 *M* sodium hydroxide. All solutions were de-aerated with helium prior to use. Optically active protected amino acids were of the L-configuration and purchased from Chemical Dynamics and BACHEM (Torrance, CA, U.S.A.) and were checked for purity by thin-layer chromatography (TLC), melting point, and optical rotation.

Methods

The HPLC system was comprised of two Constametric pumps (Models I and IIG), a Gradient Master, and a Spectromonitor III variable-wavelength UV detector linked to a Model 3400 Recorder (all from Laboratory Data Control, Riviera Beach, FL, U.S.A.). Samples were introduced through a Rheodyne injector No. 7125 (Rheodyne, Cotali, CA, U.S.A.). The analytical columns [Synchropak RP-P C_{18} (25 × 0.41 cm or 25 × 1 cm)] (SynChrom, Linden, IN, U.S.A.) were used with a precolumn of Copell ODS pelicular packing (Whatman, Clifton, NJ, U.S.A.). The unretarded peaks correspond to the presence of ammonium acetate, sodium chloride

										10			
rIFN-αA	Cys	Asp	Leu	Pro	Gln	Thr	His	Ser	Leu	Gly	Ser	Arg	Arg
rIFN-αD	"	"	"	"	Glu	"	"	"	"	Asp	Asn	"	"
rIFN-αA/D (Bgl II)	"	"	"	"	Gln	"	"	"	"	Gly	"	"	"
							20						
rIFN-αA	Thr	Leu	Met	Leu	Leu	Ala	Gln	Met	Arg	Lys	Ile	Ser	Leu
rIFN-αD	"	"	"	"	"	"	"	"	Ser	Arg	"	"	Pro
rIFN-αA/D (Bgl II)	"	"	"	"	"	"	"	"	Arg	Lys	"	"	Leu
				30									
rIFN-αA	Phe	Ser	Cys	Leu	Lys	Asp	Arg	His	Asp	Phe	Gly	Phe	Pro
rIFN-αD	Ser	"	"	"	Met	"	"	"	"	"	"	"	"
rIFN-αA/D (Bgl II)	Phe	"	"	"	Lys	"	"	"	"	"	"	"	"
												50	
rIFN-αA	40 Gln	Glu	Glu	Phe		Gly	Asn	Gln	Phe	Gln	Lys	Ala	Glu
rIFN-αD	"	"	"	"	Asp	"	"	"	"	"	"	"	Pro
rIFN-αA/D (Bgl II)	"	"	"	"		"	"	"	"	"	"	"	Glu
									60				
rIFN-αA	Thr	Ile	Pro	Val	Leu	His	Glu	Met	Ile	Gln	Gln	Ile	Phe
rIFN-αD	Ala	"	Ser	"	"	"	"	Leu	"	"	"	"	"
rIFN-αA/D (Bgl II)	Thr	"	Pro	"	"	"	"	Met	"	"	"		"
						70							
rIFN-αA	Asn	Leu	Phe	Ser	Thr	Lys	Asp	Ser	Ser	Ala	Ala	Trp	Asp
rIFN-αD	"	"	"	Thr	"	"	"	"	"	"	"	"	"
rIFN-αA/D (Bgl II)	"	"	"	"	"	"	"	"	"	"	"	"	"
			80										90
rIFN-αA	Glu	Thr	Leu	Leu	Asp	Lys	Phe	Tyr	Thr	Glu	Leu	Tyr	Gln
rIFN-αD	"	Asp	"	"	"	"	"	Cys	"	"	"	"	"
rIFN-αA/D (Bgl II)	"	"	"	"	"	"	"	"	"	"	"	"	"
										100			
rIFN-αA	Gln	Leu	Asn	Asp	Leu	Glu	Ala	Cys	Val	Ile	Gln	Gly	Val
rIFN-αD	"	"	"	"	"	"	"	"	"	Met	"	Glu	Glu
rIFN-αA/D (Bgl II)	"	"	"	"	"	"	"	"	"	"	"	"	"
							110						
rIFN-αA	Gly	Val	Thr	Glu	Thr	Pro	Leu	Met	Lys	Glu	Asp	Ser	Ile
rIFN-αD	Arg	"	"	"	Gly	"	"	"	Asn	Val	"	"	"
rIFN-αA/D (Bgl II)	"	"	"	"	"	"	"	"	"	"	"	"	"
				120									
rIFN-αA	Leu	Ala	Val	Arg	Lys	Tyr	Phe	Gln	Arg	Ile	Thr	Leu	Tyr
rIFN-αD	"	"	"	Lys	"	"	"	Arg	"	"	"	"	"
rIFN-αA/D (Bgl II)	"	"	"	"	"	"	"	"	"	"	"	"	"
	130										140		
rIFN-αA	Leu	Lys	Glu	Lys	Lys	Tyr	Ser	Pro	Cys	Ala	Trp	Glu	Val
rIFN-αD	"	Thr	"	"	"	"	"	"	"	"	"	"	"
rIFN-αA/D (Bgl II)	"	"	"	"	"	"	"	"	"	"	"	"	"
								150					
rIFN-αA	Val	Arg	Ala	Glu	Ile	Met	Arg	Ser	Phe	Ser	Leu	Ser	Thr
rIFN-αD	"	"	"	"	"	"	"	"	Leu	"	"	"	"
rIFN-αA/D (Bgl II)	"	"	"	"	"	"	"	"	"	"	"	"	"
					160					165			
rIFN-αA	Asn	Leu	Gln	Glu	Ser	Leu	Arg	Ser	Lys	Glu			
rIFN-αD	"	"	"	"	Arg	"	"	Arg	"	"			
rIFN-αA/D (Bgl II)	"	"	"	"	"	"	"	"	"	"			

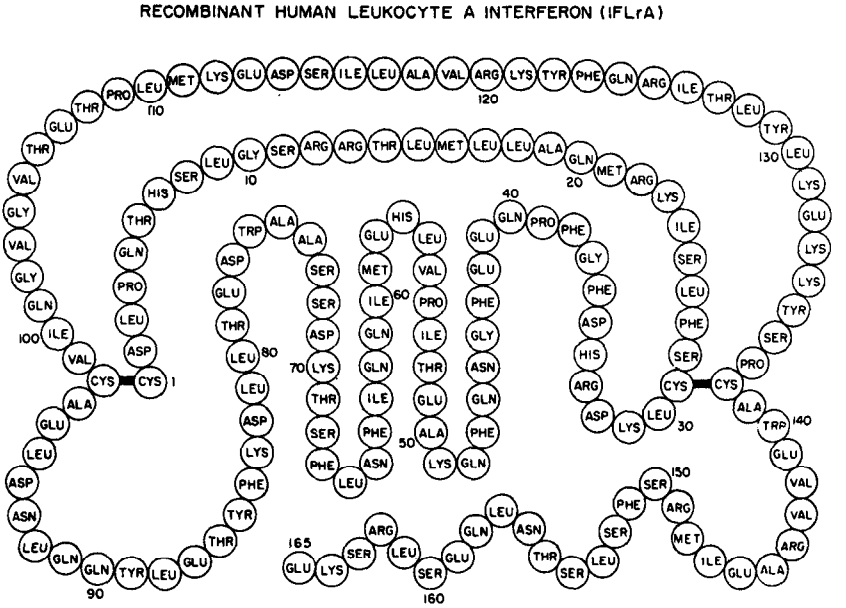


Fig. 1. Summary of the sequences of the recombinant human leukocyte interferons IFN-αA, IFN-αD and the hybrid IFN-αA/D (Bgl II). The entire sequence of rIFN-αA is shown. The amino acids of the rIFN-αD and rIFN-αA/D are shown only where they differ from the sequence of rIFN-αA.

and TWEEN-20 present in the samples. Preparative HPLC was performed on Whatman Partisil Magnum-9 (50 × 0.94 cm) or Magnum 20 (50 × 2 cm) ODS-3 columns. PAGE was performed as described by Laemmli[19] in a Hoefer Vertical Gel Unit SE-500 (Bio-Rad Labs., Richmond, CA, U.S.A.).

The protected amino acids were examined by TLC on Silica Gel G plates (Analtech, Newark, NJ, U.S.A.) and developed with chlorine-4,4'-tetramethyldiaminodiphenylmethane (TDM). Their melting points (uncorrected) were determined on the Thomas-Hoover apparatus (Thomas, Philadelphia, PA, U.S.A.), and their optical rotations were measured in a jacketed 1-dm cell on a Perkin-Elmer (Norwalk, CT, U.S.A.) Model 141 polarimeter and conformed to the accepted values. For amino acid analyses the peptides were hydrolyzed for 24 h in 6 *N* hydrochloric acid, containing 1% thioglycolic acid. For difficult cleavages longer times or higher temperatures were used. Analyses were performed on the Beckman Model 121M amino acid analyzer (Beckman, Berkeley, CA, U.S.A.).

Synthesis

rIFN-α (and oligomers), rIFN-αD and the rIFN-αA/D hybrid were prepared and purified as previously described[3,5,15]. Syntheses were carried out with the Vega Model 250 Peptide Synthesizer (Vega, Tucson, AZ, U.S.A.). (For an excellent review in the solid-phase peptide synthesis methodology, see ref. 20.) Trifunctional amino acids were protected as N^{α}-Boc-Arg(Tos), N^{α}-Boc-Asp(OBzl), N^{α}-Boc-Glu(OBzl), N^{α}-Boc-Lys(2-Cl-Z), N^{α}-Boc-Ser(Bzl), N^{α}-Boc-Thr(Bzl), N^{α}-Boc-Tyr(2,6-Cl_2-Bzl), and N^{α}-Boc-Trp(For). (Nomenclature and abbreviations follow the recommendations of the IUPAC-IUB Commission[21].)

Peptide synthesis was performed in a stepwise fashion. Double couplings were carried out by the performed symmetric anhydride procedure[22]. All solvents were used in a ratio of 15–20 ml/g of resin. The protocol for a typical cycle was as follows: (1) TFA–dichloromethane (1:1) for 1 min, (2) TFA–dichloromethane (1:1) for 30 min, (3) dichloromethane 4 times for 1 min, (4) DIEA–dichloromethane (8:92) for 2 min, (5) dichloromethane for 1 min, (6) repeat steps 4 and 5, (7) 2-propanol twice for 1 min, (8) dichloromethane 6 times for 1 min, (9) 3 equiv. of preformed symmetric anhydride of Boc-amino acid for 20 min, (10) DIEA–dichloromethane (1:99) for 10 min, (11) dichloromethane once for 2 min, (12) repeat steps 9, 10, 11, and (13) dichloromethane 6 times for 2 min. Exception to this protocol was the coupling of Boc-Asn (6 equiv.) in DMF by using DCC and 1-hydroxybenzotriazole[23]. Coupling efficiency was monitored after every cycle by the ninhydrin method. Whenever the ninhydrin test was still slightly positive, even after three couplings, the remaining unreacted amino groups were acetylated with acetic anhydride–pyridine.

IFN-αD(140–166)

The synthesis of IFn-αD(140–166) was carried out by using the phenylacetamidomethyl-resin modification[24] by the procedures outlined above. The peptide–resin was cleaved with hydrofluoric acid by using the "low–high" modification[25] to give crude peptide (Fig. 6A). Purification by preparative HPLC on a Whatman Partisil Magnum 20 ODS-3 column (50 × 2 cm) and elution with a water (0.1% TFA)–acetonitrile (0.1% TFA) gradient gave a homogeneous product (Fig. 6B). Amino acid analysis: Asp, 1.00; Thr, 0.96; Ser, 2.84; Glu, 5.18; Gly, 1.02; Ala, 2.02; Val, 1.96; Met, 0.94; Ile, 0.96; Leu, 4.10; Lys, 1.00; Arg, 4.14; Trp, 1.05.

IFN-αA(105–125)

The synthesis and purification of IFN-αA(105–125) was achieved in the same general manner to give a product which was homogeneous by analytical HPLC (Fig. 7A). Amino acid analysis: Asp, 1.07; Thr, 1.82; Ser, 0.96; Glu, 3.12; Pro, 0.89; Ala, 1.08; Val, 1.79; Met, 0.72; Ile, 0.87; Leu, 2.00; Tyr, 1.02; Phe, 1.07; Lys, 2.07; Arg, 1.94.

IFN-αA[Val^{113}](105–125)

Synthesis and purification of IFN-αA[Val^{113}](105–125) was carried out by the same general method to give a product which was homogeneous by analytical HPLC (Fig. 8A). Amino acid analysis: Asp, 1.09; Thr, 1.89; Ser, 0.93; Glu, 2.03; Pro, 0.95; Ala, 1.11; Val, 2.73; Met, 0.92; Ile, 0.99; Leu, 1.96; Tyr, 1.08; Phe, 1.06; Lys, 2.06; Arg, 2.10.

IFN-αA[Δ^3 Pro^{109}, Val^{113}](105–125)

The synthesis and purification of IFN-αA[Δ^3 Pro^{109}, Val^{113}](105–125) was carried out in the same general manner to give a product which was homogeneous by analytical HPLC (Fig. 8B). Amino acid analysis: Asp, 1.01; Thr, 1.77; Ser, 0.91; Glu, 1.92; Pro, 0.95; Ala, 1.09; Val, 2.56; Met, 1.05; Ile, 1.03; Leu, 1.98; Tyr, 0.98; Phe, 1.08; Lys, 2.08; Arg, 2.04.

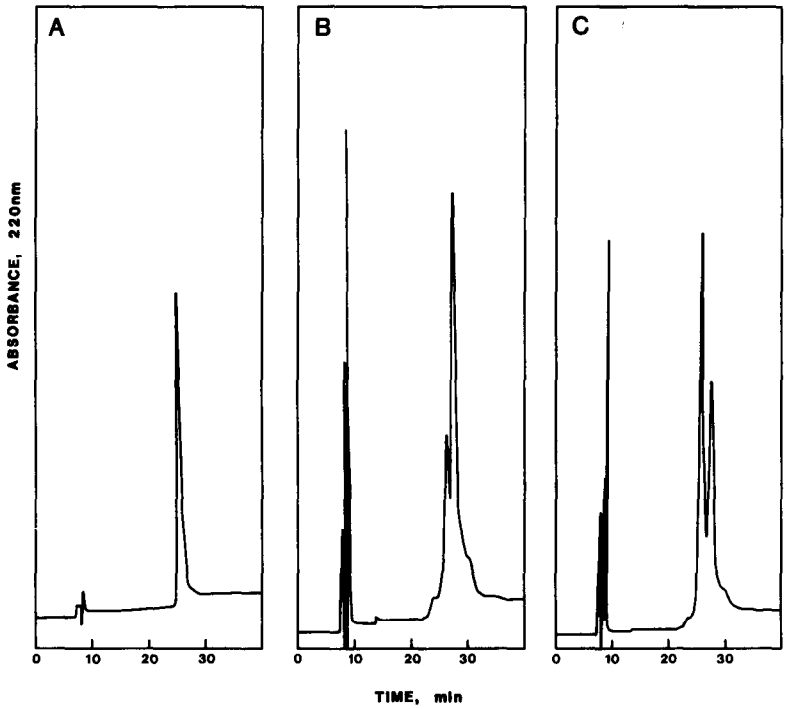


Fig. 2. HPLC analysis of (A) 16.4 μg of rIFN-αA [purified, fast-moving monomer (FMM)]; (B) 40 μg of rIFN-αA [partially reduced slow-moving monomer (SMM)]; (C) mixture of rIFN-αA (16.4 μg of FMM and 26.6 μg of SMM) on a Synchropak RP-P C_{18} (25 × 1 cm) column eluted with (a) water (0.025% TFA) to (b) acetonitrile (0.025% TFA) with a linear gradient (30–60% b) in 30 min. Detection, 220 nm; flow-rate, 2 ml/min; sensitivity, 0.2 a.u.f.s.

RESULTS AND DISCUSSION

Purified rIFN-αA with both disulfide bonds intact (FMM) was found to be homogeneous by HPLC (Fig. 2A). This is in agreement with the detection of one major band by SDS-PAGE (Fig. 3, lane 5) [compare to partially purified FMM (Fig. 3, lane 2)]. Analytical HPLC of SMM revealed the presence of one major and one minor component (Fig. 2B) which was not as clearly resolved by SDS-PAGE (Fig. 3, lane 4). Admixture of FMM and SMM (Fig. 2C) showed that the earlier peak in SMM did not correspond to FMM and led to the conclusion that FMM is not present in SMM.

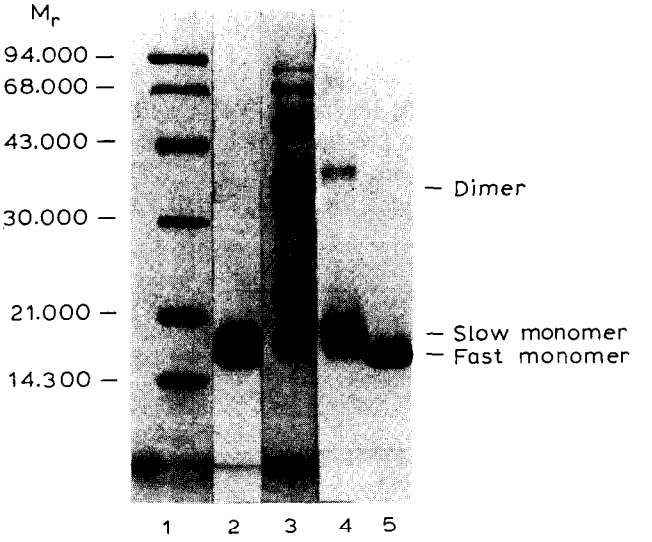


Fig. 3. SDS-PAGE of leukocyte interferon molecules. Migration of the molecular weight standards is shown in lane 1; lane 2, partially purified FMM; lane 3, partially purified oligomeric mixture; lane 4, purified SMM, lane 5, purified FMM.

Purified rIFN-αD and the hybrid rIFN-αA/D were clearly separated from each other by HPLC as well as from rIFN-αA (Fig. 4). Oligomers of rIFN-αA are known to consist of dimers, trimers, etc., containing intermolecular disulfide bonds involving Cys^1 and Cys^{98} (see Fig. 1), (*e.g.* Cys^1–Cys^1, Cys^1–Cys^{98}, Cys^{98}–Cys^{98})[8]. Separation of these oligomers can be carried out by SDS-PAGE (Fig. 3, lane 3) or by HPLC with the 0.1% perchloric acid (pH 2.5)–acetonitrile gradient system. These oligomers were shown by HPLC to be complex mixtures containing numerous components. The earliest oligomeric peak in Fig. 5A (approx. 20.5 min), present in only minor amounts, was shown to correspond to FMM by admixture studies (Fig. 5D). The dimers (Fig. 5B) and trimers (Fig. 5C) can each be resolved into several components, corresponding to the various combinations of disulfide bonds, and are readily distinguished from the monomeric forms of rIFN-αA.

The large-pore (300 Å) Synchropak RP-P C_{18} column was also found to be useful for the separation of IFN-αA and IFN-αD peptide fragments. An IFN-αD

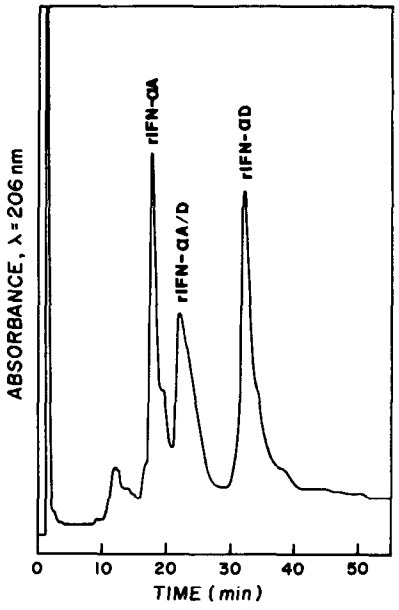


Fig. 4. HPLC of mixture of 15 μg of rIFN-αA, 8 μg of rIFN-αA/D, and 15 μg of rIFN-αD on a Synchropak RP-P C_{18} (25 × 0.42 cm) column, eluted with (a) 0.1% perchloric acid (pH 2.5) to (b) acetonitrile with a linear gradient (48–60% b) in 45 min. Detection at 206 nm; flow-rate 2 ml/min; sensitivity, 0.1 a.u.f.s.

peptide fragment comprising residues (140–166) was synthesized and the crude product (Fig. 6A) was purified by preparative HPLC to homogeneity, as shown (Fig. 6B) by analytical HPLC. IFN-αA(105–125) emerged in 24 min (Fig. 7A), while IFN-αA[Val^{113}](105–125) emerged in 28 min (Fig. 7B). Remarkably, this system was also found to be capable of distinguishing IFN-αA[Val^{113}](105–125) (Fig. 8A) from

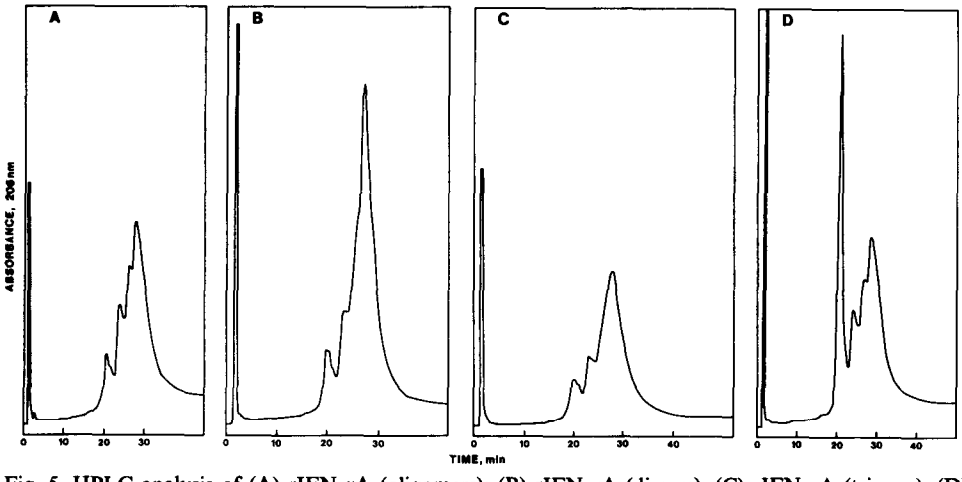
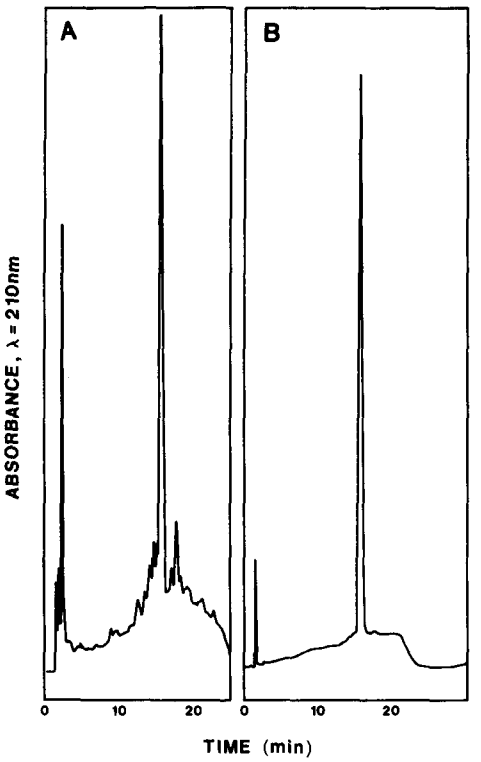


Fig. 5. HPLC analysis of (A) rIFN-αA (oligomers); (B) rIFN-αA (dimers); (C) rIFN-αA (trimers); (D) mixture of rIFN-αA (oligomers) with FMM on a Synchropak RP-P C_{18} (25 × 0.41 cm) column, eluted with (a) 0.1% perchloric acid (pH 2.5) to (b) acetonitrile with a linear gradient (35–50% b) in 30 min. Detection at 206 nm; flow-rate 2 ml/min; sensitivity, 0.1 a.u.f.s.



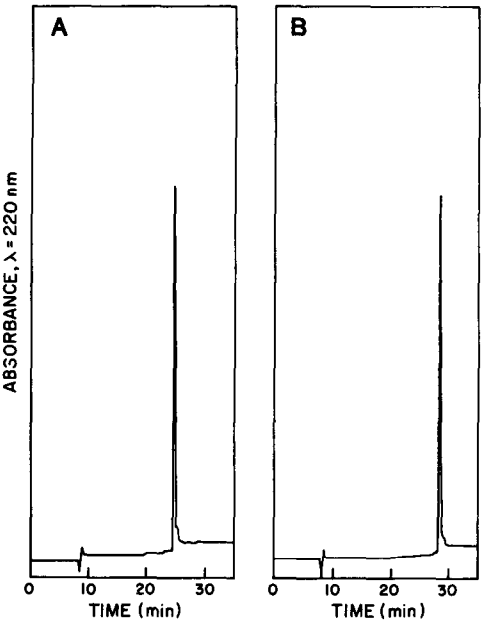


Fig. 6. HPLC of IFN-αD(140–166); (A) 50 μg of crude and (B) 7.5 μg of purified on a Synchropak RP-P C_{18} column (25 × 0.41 cm), eluted with (a) water (0.025% TFA) to (b) acetonitrile (0.025% TFA) with a linear gradient (20–45% b) in 30 min. Detection at 210 nm; flow-rate, 2 ml/min; sensitivity 0.2 a.u.f.s.

Fig. 7. HPLC of IFN-αA peptide fragments: (A) 20 μg of IFN-αA(105–125) and (B) 20 μg of IFN-αA[Val^{113}](105–125) on a Synchropak RP-P C_{18} column (25 × 1 cm), eluted with (a) water (0.025% TFA) to (b) acetonitrile (0.025% TFA) with a linear gradient (15–40% b) in 30 min. Detection at 220 nm; flow-rate, 2 ml/min; sensitivity, 0.2 a.u.f.s.

IFN-αA[Δ^3 Pro^{109}, Val^{113}](105–125) (Fig. 8B) (see admixture chromatogram, Fig. 8C).

Analytical HPLC on the Synchropak RP-P C_{18} (300 Å) system is a powerful tool for the evaluation of rIFN-αA, rIFN-αA/D, and related synthetic peptide fragments. These chromatograms established the general utility of our method for separating (a) monomeric and oligomeric forms of rIFN-αA, (b) different recombinant interferon species and (c) peptide fragments of both IFN-αA and IFN-αD.

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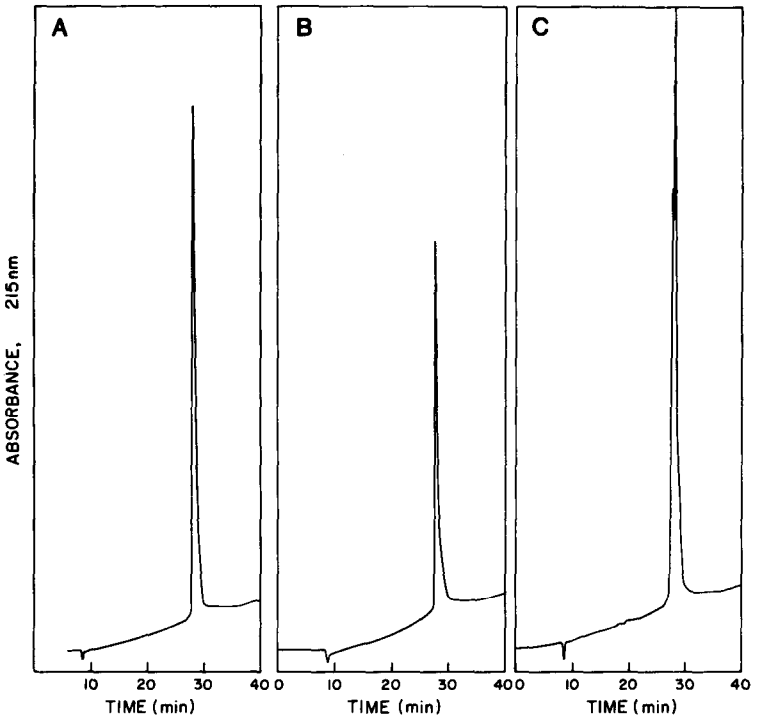

Fig. 8. HPLC of IFN-αA peptide fragments: (A) 20 μg of IFN-αA[Val^{113}](105–125), (B) 15 μg of IFN-αA[Δ^3Pro^{109}, Val^{113}](105–125), and (C) admixture of 15 μg [Δ^3Pro^{109},Val^{113}](105–125) and 20 μg IFN-αA[Val^{113}] (105–125) on a Synchropak RP-P C_{18} (25 × 1 cm) column eluted with (a) water (0.025% TFA) to (b) acetonitrile (0.025% TFA) with a linear gradient (15–40% b) in 30 min. Detection at 215 nm; flow-rate, 2 ml/min; sensitivity, 0.2 a.u.f.s.

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