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The inactivation process of fibroblast interferon-β in the preparation stage and in rabbit nasal absorption of mixed and freeze-dried powder dosage forms

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Summary

The present paper is concerned with a new finding with regard to the inactivation and the nasal bioavailability of IFN-β in two different powder dosage forms, i.e. mixed powder (MP) and freeze-dried powder (FP) dosage forms. The two types of preparations which contained bile salts were prepared. The IFN-β titer was determined by bioassay and enzyme immunoassay (EIA). Employing EIA, it was confirmed that the titers of sample MP and sample FP after preparation were different, while their bioavailabilities in the nasal absorption study were not significantly different. In an attempt to clarify the above result, studies of stability and rabbit nasal absorption of IFN-β were performed, additionally investigating the effects of cryoprotectants for sample FP. As a result, in the case of sample MP, rapid inactivation seemed to take place mainly on the nasal mucosal membrane during the absorption stage, while for sample FP it appears mostly during the preparation stage on freeze-drying. Additionally, the pharmacokinetic behavior of IFN-β was found to differ between both samples.

Introduction

Human fibroblast interferon-β (IFN-β), an antiviral and antineoplastic substance, is now clinically administered only through intravenous injection. Since the method of intravenous injection is inconvenient and uncomfortable for patients, it is desirable to provide other routes of administration.

In general, peptides are usually inactivated when administered orally due to the presence of various peptidases in the gastrointestinal tract and extensive hepatic first-pass elimination. Therefore, the search for alternative methods of administration of biomedicals such as peptides is becoming more intense. The administration of interferon by routes other than injection has been investigated previously, for example, via oral (Cantell et al., 1973; Panlesu et al., 1988), intradermal (Bocci et

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al., 1986), colorectal (Bocci et al., 1985) and nasal (Douglas et al., 1974; Maitani et al., 1986; Igawa et al., 1988) administration.

With regard to the nasal administration of IFN-β, in addition to the general systemic effect, it may also exert a prophylactic effect on common viral upper respiratory tract infection (Douglas et al., 1974). As one of the merits of nasal administration of IFN-β, the first-pass effect may be avoided and thus provide a more convenient method for self-medication by patients, which is preferable for the long-term medication of biological response modifier such as IFN-β. On the other hand, there are disadvantages such as excessively rapid absorption intravenously, side-effects on the function of smell, and variation in bioavailability through different means of administration.

Considering both the advantages and disadvantages, we performed several investigations on the nasal administration of IFN-β from the pharmaceutical point of view. The present paper is concerned with a new finding regarding the inactivation and nasal bioavailability of IFN-β in two different powder dosage forms, i.e. mixed powder (MP) and freeze-dried one (FP).

Both kinds of dosage form, which contained absorption promoters, were prepared and subjected to analysis since it had been reported previously that IFN alone is absorbed neither in solution nor in powder dosage forms (Maitani et al., 1986). The IFN-β titer was determined by bioassay and enzyme immunoassay (EIA) (Igawa et al., 1986). Employing EIA, it was confirmed that the titers of sample MP and sample FP after preparation were different while their bioavailabilities in the absorption study were not significantly different.

In an attempt to clarify the above result, analysis of the stability and rabbit nasal absorption was performed on IFN-β, additionally investigating the effects of cryoprotectants for sample FP.

Materials and Methods

Chemicals

The IFN-β used was a preparation from Toray Industries (Tokyo, Japan, 3×10^6 international units (IU) per vial; 1 dose vial). The sources of the materials used as bile salts and cryoprotecting excipients were as follows: glycocholate (GC), glycodeoxycholate (GDC), taurodeoxycholate (TDC), taurocholate (TC), deoxycholate (DC), and cholate (C) (in each case, as the sodium salt; Sigma, St. Louis, MO, U.S.A.). Sucrose, maltose, trehalose (Wako, Osaka, Japan), D-glucuronic acid (Nakarai, Kyoto, Japan), glyceric acid and human serum albumin (HSA, Chem. & Serum Therapy Lab., Japan).

Preparation of sample MP and FP

The sample MP was prepared as follows: To one dose vial of IFN-β, also containing 3 mg of HSA as the stabilizer, 3 mg of bile salt and 13.74 mg of HSA were added, then mixed and passed through a 100 mesh sieve.

The sample FP was prepared by dissolving one dose vial of IFN-β also containing 3 mg of HSA as the stabilizer, together with 3 mg of bile salts (GC and TC) and 13.74 mg of HSA into 2 ml of distilled water (pH-unadjusted case) or 11.61 mg of HSA with 10 mM citrate buffer (pH 5.5) into 2 ml of distilled water (pH-adjusted case), followed by freeze-drying for 24 h. The preparation was administered after sieving through 100 mesh.

Nasal administration of the powder dosage form and intravenous injection

Male New Zealand White rabbits (Saitama Experimental Animal Supply Co.; 3.0–3.6 kg) were used. Rabbits were fasted for 20 h before intranasal administration. The apparatus for the nasal administration of the powder dosage form consisted of a special sprayer (i.e. rubber bulb with reservoir), an Eppendorf pipette tip and polyethylene tubing. The sample powder was placed in the Eppendorf pipette tip which was connected to the polyethylene tubing (1.20 mm i.d. and 1.70 mm o.d.). The tubing was inserted into the nasal cavity at a position about 2.0 cm from the nostril and the powder was sprayed in.

The dose was 3×10^6 IU/rabbit for nasal administration. For intravenous injection, each rabbit received 3×10^6 IU through the marginal vein of the left ear.

Collection of blood samples

Blood samples (2.0 ml) were collected in silicon-coated glass tubes by cutting the vena auricularis with a Blood Lance (Becton & Dickinson, U.S.A.) immediately prior to and at 0.25, 0.5, 1, 3, 6 and 24 h after nasal administration of IFN-β. Serum was separated by centrifugation at $1500 \times g$ for 15 min. The serum samples were stored at $-70\,^{\circ}$C until analysis.

Determination of titer of preparation and serum concentration of IFN-β

The IFN-β titer of preparations of samples MP, FP and other test samples was determined by both EIA and bioassay in order to ensure measurement of the intrinsic activity. Serum IFN-β was assayed by EIA.

In EIA, microplate wells were coated with anti-rabbit IFN-β antibody as the first antibody. The enzyme reaction was determined by recording the change in absorbance. The amount of IFN-β in samples was calculated using standard curves obtained from reference IFN-β which had been standardized against the international reference for IFN-β (G-023-902-527, NIH, Bethesda, MD) by bioassay (Yamazaki et al., 1988).

In the bioassay, a dye binding method was carried out by using FL cells and Sindbis virus. The IFN-β titer was evaluated by the technique based on determining the 50% cytopathic effect reduction and expressed in international units (IU) with respect to the international reference preparation of IFN-β. The minimum detectable levels by bioassay and EIA were found to be 10 and 5 IU/ml, respectively, for plasma in rabbits.

Data analysis

The pharmacokinetic parameters after both intravenous and intranasal administration of IFN-β were calculated using the MULTI computer program in which the Simplex method was incorporated, and applying a two-compartment model with intravenous injection and a one-compartment model with nasal absorption, respectively (Tanigawara et al., 1983). The simulated curves from the model calculations were found to yield a good fit to the data (Maitani et al., 1989). The area under the serum IFN-β concentration-time curve (AUC_0^∞) was determined by curve fitting in the case of intravenous administration, and via the trapezoidal formula from 0 to 6 h, and extrapolation using an exponential function after 6 h in the intranasal administration. The absolute bioavailability (F) following intranasal administration was determined by using Eqn 1:

$$F = AUC_0^\infty(\text{nasal})/AUC_0^\infty(\text{i.v.}) \quad (1)$$

Stability study of IFN-β with cryoprotecting excipients in citrate buffer

IFN-β solutions were prepared with 1 ml of 10 mM citrate buffer, 9 mg of various cryoprotecting excipients, 3 mg of GC and 1 ml of IFN-β solution (3×10^6 IU). For the control, an IFN-β solution which contained 1 ml of 10 mM citrate buffer, 12 mg of various cryoprotecting excipients and 1 ml of IFN-β solution (3×10^6 IU) was used.

The stability test was carried out under the following conditions including the initial: (1) incubation for 30 min and 3 h at $4\,^{\circ}$C; (2) incubation for 30 min and 3 h at $37\,^{\circ}$C; (3a) frozen for 3 h and dissolved for analysis; (3b) frozen without GC for 3 h and dissolved for analysis.

For the analysis, the solutions were frozen at $-80\,^{\circ}$C for 3 h, after which they were thawed in water. 100 μl samples of the solutions were taken and added to 900 μl of 5 % Eagle culture containing fetal calf serum. The samples were stored at $-30\,^{\circ}$C until analysis.

Results and Discussion

Effect of bile salts on nasal absorption of sample MP

In order to find an effective absorption promoter for nasal absorption of IFN-β of the powder samples, the effect of bile salts on sample MP was examined, the results being listed in Table 1. Trihydroxy bile salts (C, GC and TC) enhanced the bioavailability (F) more extensively than the dihydroxy forms (DC, GDC and TDC). This order of bile salt efficacy with regard to AUC was the reverse of that reported previously for the nasal absorption of insulin (Gordon et al., 1985).

TABLE 1

Effect of bile salts on nasal absorption of IFN-β (3×10^6 IU) of sample MP

Formula	Bile salt	*n*	$AUC_0^\infty \pm$ S.E. (IU ml h^{-1})	*F* (%)
F 1-1	C	4	86.0 ± 34.8	2.6
F 1-2	DC	3	59.6 ± 4.3	1.8
F 1-3	GC	3	161.3 ± 42.4	4.8
F 1-4	GDC	3	131.9 ± 18.7	3.9
F 1-5	TC	4	120.0 ± 21.4	3.6
F 1-6	TDC	3	49.1 ± 13.3	1.5

Dose was 1×10^6 IU/kg; $F = AUC_0^\infty$(nasal)/AUC_0^∞(i.v.)$\times$ 100. The titer of sample MP after preparation was 102.4%.

Insulin and IFN-β are considered to permeate through pores in the nasal mucosa, which are temporarily formed by the bile salts. It is known that, in general, dihydroxy bile salts are more effective in nasal absorption than the trihydroxy forms (Duchateau et al., 1986). However, in the present case of IFN-β, the enhancing effect of trihydroxy bile salts was higher as compared with that of the dihydroxy forms.

Therefore, it appears that the difference in effect of the various bile salts on nasal absorption of insulin and IFN-β may be influenced by the chemical properties of insulin and IFN-β. In our experiments, the IFN-β contains HSA in order to ensure its stability. HSA may exert a significant effect on the chemical properties of IFN-β.

Two of the bile salts, i.e., GC and TC, which were effective in enhancing the nasal absorption of IFN-β for the present sample MP, were also used with sample FP, as described below. The composition of sample FP is shown in Table 2.

TABLE 2

Composition of sample FP of IFN-β with GC and TC

Formula	IFN-β (ml)	GC (mg)	TC (mg)	C.B. (ml)	HSA (mg)
F 2-1	1	3		1	11.61
F 2-2	1	3			13.74
F 2-5	1		3	1	11.61
F 2-6	1		3		13.74

GC, glycocholate (sodium); TC, taurocholate (sodium); C.B., 10 mM citrate buffer (pH 5.5); HSA, human serum albumin.

Inactivation by bile salts and stabilization by cryoprotectants of IFN-β in sample FP during the preparation stage

As shown in Fig. 1, the titer of sample MP (formula F 1-3) after preparation did not decrease, amounting to 102.4%. On the other hand, the titer of sample FP in both the pH-adjusted and unadjusted case (formulae F 2-1 and 2-2) decreased to about one-third of the initial IFN-β after preparation, showing the values of 34.7 and 26.5%, respectively.

However, when these samples were administered in rabbits, the bioavailability of both MP and FP samples was almost equal, i.e., 4.8 and 4.4%, respectively, as shown in Table 4.

In order to determine the step during which sample FP IFN-β was inactivated, the titer of the sample was determined immediately after dissolution and after freezing as well. There was little change in the IFN-β titer for the sample without GC being dissolved and that frozen for 3 h without dissolving GC, as shown in Fig. 2. On the other hand, in the case of the sample with GC, the titer decreased to 91.3% on being dissolved only and to 80.1% when frozen for 3 h.

GC is necessary as a promoter for nasal absorption of IFN-β, however, it results in the inactivation of IFN-β in the preparation step of sample FP, even though the sample contains HSA as cryoprotectant. Therefore, in order to find a suitable cryoprotecting excipient other than HSA to stabilize IFN-β in the preparation step, the stability of IFN-β was examined in the presence

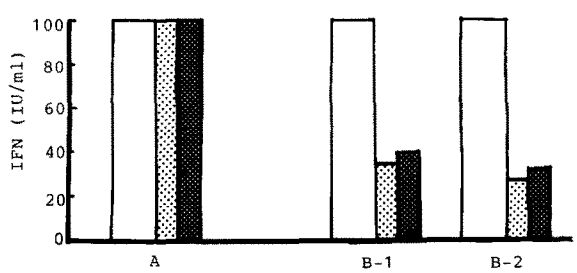

Fig. 1. Residual IFN-β titer after preparation of samples MP and FP of IFN-β with GC and HSA. (Unfilled bars) Initial, (dotted bars) EIA after preparation, (cross-hatched bars) bioassay after preparation. (A) Sample MP (formula F 1-3), (B-1) sample FP (pH-adjusted, formula F 2-1), (B-2) sample FP (pH-unadjusted, formula F 2-2).

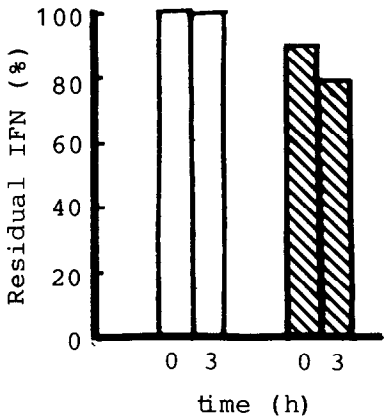

Fig. 2. Stability of IFN-β with HSA after dissolution and while frozen for 3 h in citrate buffer. (Unfilled bars) Without GC, (hatched bars) with GC.

of various polyol cryoprotecting excipients. When the incubation of IFN-β solution was performed at 4°C, the actvity of IFN-β did not decrease significantly, as shown in Fig. 3. Among the excipients tested, HSA was the most effective in maintaining the titer of IFN-β.

However, after 30 min incubation at 37°C of samples FP, which were prepared with the cryoprotectants in addition to GC, the value of the IFN-β titer determined showed the cryoprotectants to be ineffective, the titer decreasing rapidly, as shown in Fig. 4. The above results

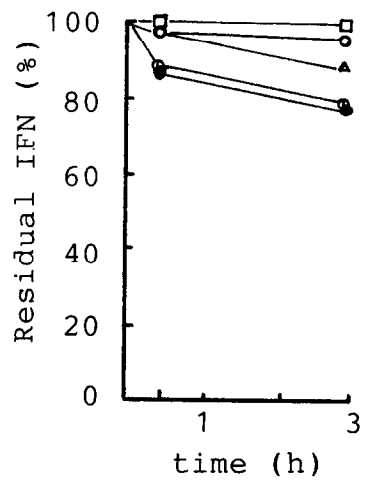

Fig. 3. Stability of IFN-β solution with GC and various excipients on incubation in citrate buffer at 4°C. (△) Sucrose, (●) maltose, (Φ) trehalose, (○) glucuronic acid, (□) HSA.

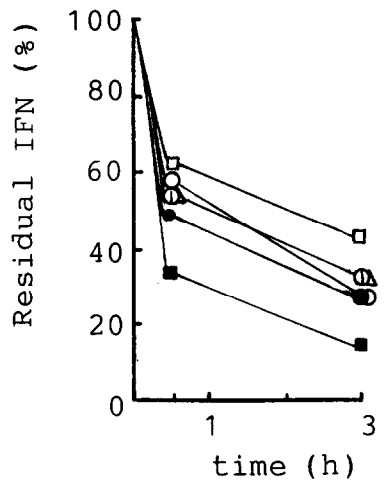

Fig. 4. Stability of IFN-β solution with GC and various excipients on incubation in citrate buffer at 37°C. (△) Sucrose, (●) maltose, (Φ) trehalose, (○) glucuronic acid, (■) glyceric acid, (□) HSA.

show that on dissolution, i.e., the first stage in the preparation of freeze-dried samples, the conditions of 4°C temperature and cryoprotectant addition may prevent the fall in the IFN-β titer, even when GC is added. After freezing of the solution for 3 h only, with DC or without GC, the titer decreased, although to a small extent only, as shown in Table 3. Thus, we have found no cryoprotectant that is more effective than HSA.

Consequently, inactivation of IFN-β of sample FP may be related to the combination of the

TABLE 3

Stability of IFN-β with various cryoprotecting excipients in the absence and presence of GC while frozen for 3 h in citrate buffer

Excipient	Residual IFN-β (%)			
	GC (−)		GC (+)	
	Initial [a]	3 h	Initial [a]	3 h
Sucrose	96.5	93.1	89.2	80.7
Maltose	98.5	95.3	92.3	76.0
Trehalose	97.8	95.0	89.2	74.3
Glucuronic acid	95.7	91.2	93.8	84.4
HSA	102.4	100.0	91.3	80.1

[a] IFN-β solution contained various excipients before freezing. Test solution contained 1 ml citrate buffer, 9 mg excipients, 3 mg GC and 1 ml IFN-β (3×10^6 IU).

processes of freezing and drying, particularly the latter, and the search for a cryoprotectant which is practically promising has been a difficult task so far.

Inactivation of IFN-β in samples MP and FP during the process from preparation to nasal absorption

As in the case of sample MP containing GC, it has been reported that a linear relationship exists between the dose and AUC_0^∞ in $1-20 \times 10^6$ IU of IFN-β (Igawa et al., 1988). If a similar relationship does exist in the present case of sample FP, the bioavailability of sample FP should amount to one-third of the dose of sample MP. The reason for this discrepancy is that sample FP was actually almost 1×10^6 IU due to inactivation during the preparation process of 3×10^6 IU (Fig. 1). However, the bioavailability of sample FP consequently equalled that of the sample MP.

Fig. 5 depicts a scheme summarizing the entire course of inactivation of IFN-β from preparation to nasal absorption. In the case of sample MP, rapid inactivation down to 62.2% takes place on a nasal mucosal membrane, as investigated previously for a suspension of homogenized mucosal membrane which was incubated for 30 min (Igawa et al., 1989).

For the sample FP, the degree of inactivation appeared to be very low, and the apparent permeability was found to be greater compared with sample MP. However, taking into consideration that the molecular weight of IFN-β is high (20000), there may be no difference in permeability constant between IFN-β in both samples. Therefore, the high apparent permeability of IFN-β in sample FP may arise from a stable conformation being adopted with the cryoprotectant HSA and/or the absorption-promoting bile salt GC in the preparation of sample FP, although the precise mechanism has not yet been established. The stable conformation of the complex that may lead to the β phase for IFN-β appears to be reversible, based on the following aspects. After preparation, the active site of IFN-β may have been modified or its conformation may have changed. If so, then the specific activity of IFN-β may decrease. After

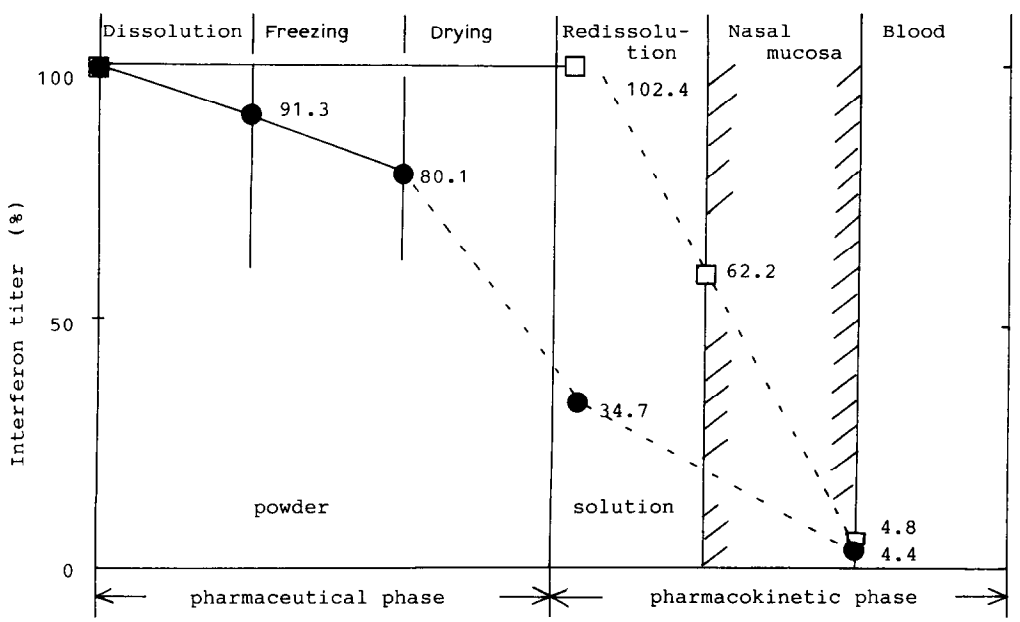

Fig. 5. Inactivation process of sample MP and FP of IFN-β from preparation to the nasal mucosa. (□) Sample MP (formula F 1-3), (●) sample FP (formula F 2-1).

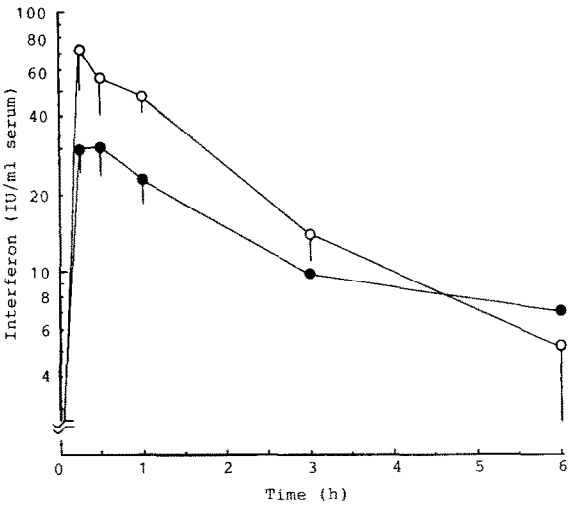

Fig. 6. Serum IFN-β levels after nasal administration with sample MP and sample FP of IFN-β (3×10^6 IU). (○) Sample MP (formula F 1-3), (●) sample FP (formula F 2-1). Values are expressed as means ± S.E. of three rabbits.

permeation of IFN-β through the nasal mucosa, it may recover its conformation and thus regain its initial activity.

The observations indicated that sample MP appeared to be stable during the preparation process, and that sample FP was stable in the process of nasal mucosal absorption.

Difference in pharmacokinetics of IFN-β between samples MP and FP in relation to that for inactivation during the nasal absorption stage

As judged on the basis of the absorption curves after nasal administration, it appeared that sample MP displayed both α and β phases, whereas sample FP showed only the β phase as demonstrated in Fig. 6. IFN-β is known to adopt two phases (α and β) in blood after intravenous administration and its activity in the β phase is very stable (Vilcek et al., 1980).

Therefore, IFN-β levels in the serum with sample FP were comparatively low, and decreased slowly. Nevertheless, the pharmacokinetic parameters, mean residence time (MRT) and elimination rate constant (K_e), exhibit greater values for sample FP than for sample MP, as shown in Table 4.

These findings may reflect a different conformation of IFN-β after preparation, which may bring about the difference in inactivation during the nasal absorption stage.

Conclusion

From the results obtained here, it was found that, during the entire process from preparation to nasal mucosal absorption, both samples follow different courses of inactivation of IFN-β. In the case of sample MP, rapid inactivation appears to take place mainly on the nasal mucosal membrane during the absorption stage, while sample FP gives rise to this phenomenon mostly during the preparation stage of freeze-drying. Additionally, it was found that the pharmacokinetics of IFN-β is different between the two samples.

If it becomes possible to prepare sample FP of 100% titer, then 12.9% bioavailability would be the result. Therefore, a trial to enhance the titer in the preparation may afford a practical means for the treatment of disease. The finding of an effective cryoprotectant bringing about the enhancement of the titer of sample FP is desirable.

TABLE 4

Pharmacokinetic parameters after nasal administration of samples MP and FP of IFN-β (all data expressed as means ± S.E.)

Sample	Formula	n	AUC_0^∞ (IU h ml^{-1})	MRT (h)	K_a (h^{-1})	K_e (h^{-1})	F (%)
Sample MP	F 1-3	3	161.33 ±42.37	2.51 ±0.25	37.15 ±9.40	0.55 ±0.08	4.8 ±1.3
Sample FP	F 2-1	3	147.35 ±12.15	8.19 ±0.92	24.72 ±9.48	0.35 ±0.07	4.4 ±0.3

Further investigations based on the present data are required in order to develop a powder dosage form of IFN-β which shows greater bioavailability in nasal administration.

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