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Determination of human interferons α, β and γ in plasma and/or pharmaceuticals

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Summary

The fact that human interferon is inactivated with proteinase such as trypsin and pepsin has been noted, and a new method for quantitative determination was established. It was revealed that the decomposing substance of the human interferon obtained by an action upon kallikrein or trypsin, under fixed conditions, hydrolyzed a substrate peptide MCA. By this method, a sensitive new method for quantitative determination of favorable reproducibility in human interferon preparations and in human plasma was established.

Introduction

The existence of interferon was not recognized until it was revealed that interferon inhibits the proliferation of villi. This phenomenon is the basis for the quantitative determination of interferon. This biological determination method is a modification of the original technique of Isaacs and Lindenmann (1957). In this method, techniques detecting various cells, villi and proliferation of villi are used. However, these quantitative determination methods have disadvantages; that is, they are delicate and require a great deal of skill and time. Their sensitivities have also improved gradually, but it is difficult to detect extremely small quantities of interferon. Chemical isolation methods and immunological determination methods for detecting interferon and determining extremely small quantities of it are expected to develop in future.

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Although it is already known that interferon is inactivated with trypsin and pepsin (Lockart, 1973), the authors noted an advantageous application of this fact and dissolved interferon with proteinase. From the fact that its decomposing substance hydrolyzed a certain substrate, the authors found a method for quantitative determination of extremely small quantities of interferon in preparations and/or body fluids (strictly speaking, an interferon-like substance).

Materials and Methods

Reagents

Interferon-α (1.0×10^6 IU, Kyoto Red Cross Blood Center). Interferon-β (1.0×10^6 IU, Mochida Pharmaceuticals, Tokyo). Interferon-γ (8000 IU, Kyoto Prefectural University of Medicine, Department of Microbiology).

Kallikrein (Bayer AG, Leverkusen, F.R.G.). Trypsin (Sigma Chemicals, St. Louis, MO, U.S.A.). Trypsin inhibitor (Sigma), aprotinin (Trasylol, Bayer).

Substrate (peptide-MCA) solution. Pro-Phe-Arg-MCA[1] (Peptide Institute, Minoh, Osaka) was dissolved in distilled water for injection to prepare a stock solution (5.76 mg/10 ml). A 0.1 mM substrate solution was prepared from the stock solution (pH 8.0).

Tris-HCl buffer solution (pH 8.0). A Tris-HCl buffer solution (pH 8.0) containing 100 mM NaCl and 10 mM $CaCl_2$ was prepared.

AMC stock solution 7-Amino-4-methyl coumarin (Peptide Institute) was dissolved in dimethyl sulfoxide to give a 10 mM (1.75 mg/ml) solution.

Acetic acid. 17% acetic acid was prepared and used for suspending the enzymatic reaction.

Human plasma. It was supplied from Kyoto Red Cross Blood Center.

Determination[2] *of interferon (INF)* α, β *and* γ

(1) Kallikrein method

IFN-α: 20 μl of a specimen solution were placed in a small test tube with a stopper; kallikrein (0.8 KU in 20 μl) was added to it and incubated at 37 ± 0.5°C for 4 min. Subsequently, aprotinin (1 KIU in 10 μl) was added to the mixture, and 2 ml of the substrate solution were added and incubated at 37 ± 0.5°C for 3 min. After keeping the mixture in an ice-bath for 3 min, 3 ml of 17% ACOH solution were added to stop the reaction, and the fluorescence of the solution reacted was determined.

IFN-β: 20 μl of a specimen solution and kallikrein (1.0 KU in 10 μl) were put in a small test tube with a stopper and incubated at 37 ± 0.5°C for 10 min. Subsequently, 2 ml of the substrate solution were added and incubated at 37 ± 0.5°C for 3 min. After keeping the mixture in an ice-bath for 3 min, 3 ml of 17% ACOH solution

[1] L-Prolyl-L-phenylalanyl-L-arginine 4-methyl-coumaryl-7-amide.

[2] Patent pending.

were added to stop the reaction, and the fluorescence of the reacted solution was determined.

IFN-γ: 20 μl of a specimen solution and kallikrein (1.2 KU in 30 μl) were placed in a small test tube with a stopper and incubated at 37 ± 0.5°C for 3 min. Subsequently, aprotinin (1.5 KIU in 20 μl) was added to 2 ml of the substrate solution and incubated at 37 ± 0.5°C for 3 min. After keeping the mixture in an ice-bath for 3 min, 3 ml of a 17% ACOH solution were added to stop the reaction, and the fluorescence of the reacted solution was determined.

(2) Trypsin method

IFN-α: 20 μl of a specimen solution and trypsin (2 μg in 20 μl) were put in a small test tube with a stopper and incubated at 37 ± 0.5°C for 3 min. After keeping the mixture in an ice-bath for 15 min, 2 ml of the substrate solution were added and incubated at 37 ± 0.5°C for 3 min. Subsequently, after keeping the mixture in another ice-bath for 15 min, 3 ml of a 17% ACOH solution were added to stop the reaction, and the fluorescence of the reacted solution was determined.

IFN-β: 20 μl of a specimen solution and trypsin (2 μg in 20 μl) were placed in a small test tube with a stopper and incubated at 37 ± 0.5°C for 10 min. After keeping the mixture in an ice-bath for 15 min, 2 ml of the substrate solution were added and incubated at 37 ± 0.5°C for 3 min. After keeping the mixture in another ice-bath for an additional 15 min, 3 ml of a 17% ACOH solution were added to stop the reaction, and the fluorescence of the reacted solution was determined.

IFN-γ: 20 μl of a specimen solution and trypsin (4 μg in 40 μl) were put in a test tube and incubated at 37 ± 0.5°C for 6 min. After keeping the mixture in an ice-bath for 15 min, trypsin inhibitor (2 μg in 20 μl) was added and incubated at 37 ± 0.5°C for 3 min. Subsequently, after keeping the mixture in another ice-bath for an additional 15 min, 3 ml of a 17% ACOH solution were added to stop the reaction, and the fluorescence of the reacted solution was determined.

(3) Conditions for determining the degree of fluorescence

The relative fluorescence of AMC was determined with a fluorescence spectromonitor under the following conditions: fluorescence spectromonitor, Model RF-500LC (Shimadzu Seisakusho, Kyoto); λ_{max} excitation, 353 nm; λ_{max} emission, 440 nm; sensitivity, 7.22; gain, 1.0; response, medium.

Results and Discussion

In the kallikrein method, human interferon is dissolved with kallikrein and the excessive amount of kallikrein is inactivated with aprotinin. From the fact that the decomposing substance of human interferon obtained in this way hydrolyzes a substrate, peptide-MCA, human interferon can be determined quantitatively by determining the fluorescence of the MCA produced. This is the principle of the kallikrein method. Since the chemical structures of human interferons α, β and γ differ from each other, different conditions for the treatment with kallikrein were

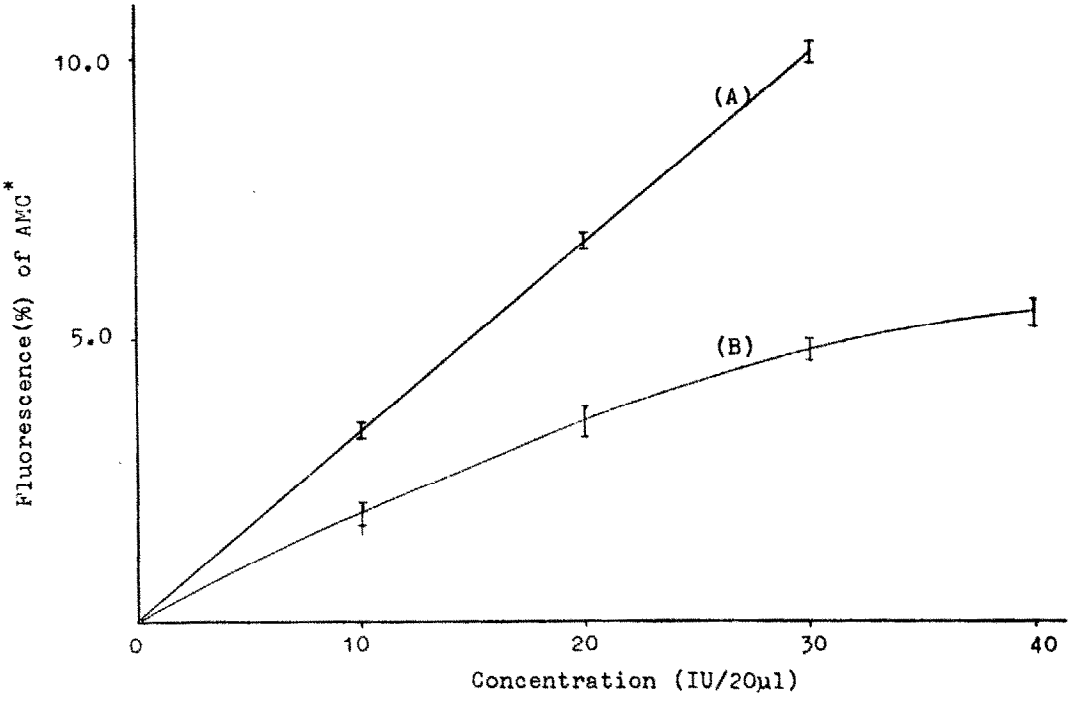

Fig. 1. Calibration curves of interferon α and β in water by using kallikrein-method (substrate: peptide-MCA **). (A) interferon α; (B) interferon β.

* 7-Amino-4-methylcoumarin.

** L-Prolyl-L-phenylalanyl-L-arginine 4-methyl-coumaryl-7-amide.

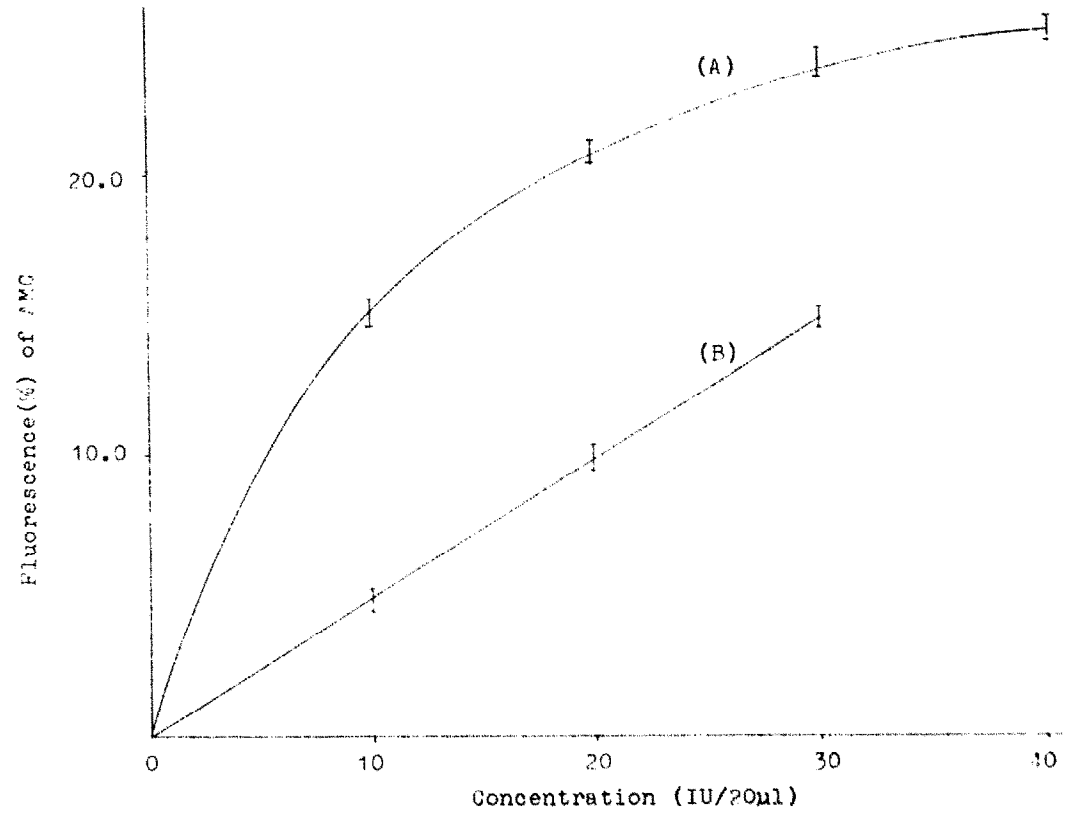

Fig. 2. Calibration curves of interferon α and β in human plasma by using the trypsin method (substrate: peptide-MCA). (A) interferon α; (B) interferon β.

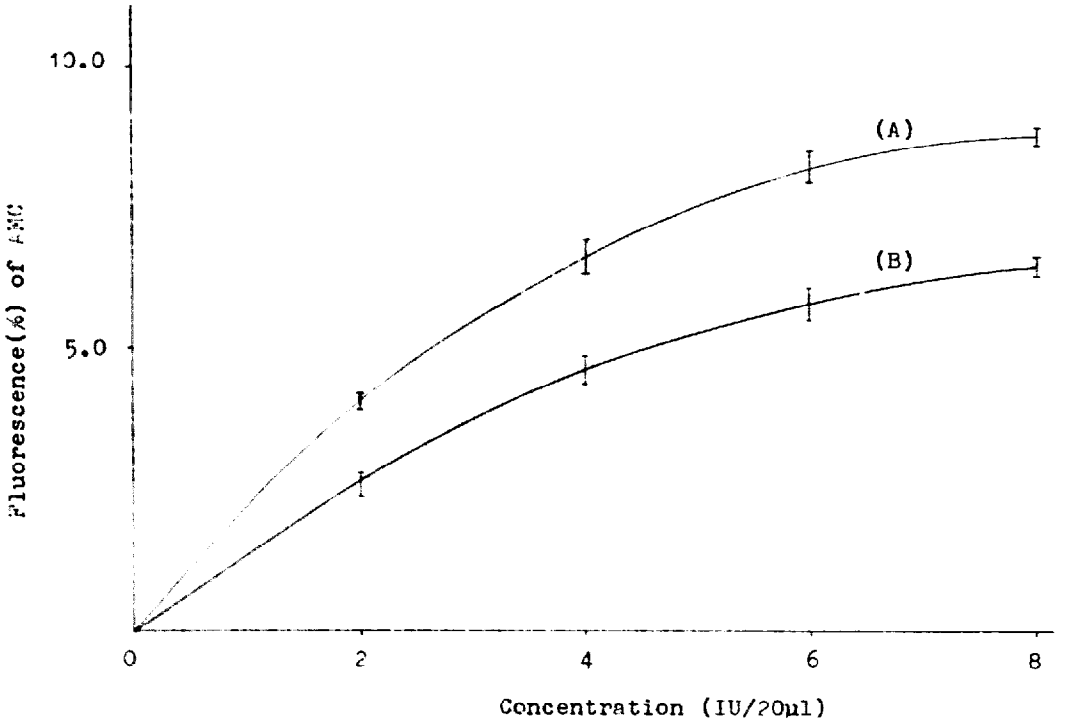

Fig. 3. Calibration curves of interferon γ in water. (A) kallikrein method (substrate: peptide-MCA). (B) trypsin method (substrate: peptide-MCA).

used in order to increase the sensitivity of the quantitative determination. Since kallikrein shows complicated changes in the living body, as previously reported by Naito et al. (1981), the kallikrein method is inappropriate for the quantitative determination of human interferon in body fluids. However, it is sufficient for determining the potency of human interferon in pharmaceutical preparations.

In the trypsin method, human interferon is dissolved with trypsin in a similar manner as in the kallikrein method, and its decomposing substance hydrolyzes the substrate, peptide-MCA. This method is characterized by favorable reproducibility, that is, human interferons not only in pharmaceutical preparations but also in body fluids can be determined quantitatively.

Calibration curves obtained by two methods in solutions and human plasma are shown in Figs. 1–3. In determining them quantitatively, conditions such as pH of buffer, incubation time, concentrations of kallikrein or trypsin, use of other proteinase for dissolving interferon, etc., were changed to select optimal conditions for the experiment. As shown in Figs. 1–3, both the kallikrein and trypsin methods showed excellent sensitivity and reproducibility, indicating that they are practically useful. As for the trypsin method, the results obtained by this method for determining blood concentration by administering human interferon to rabbits will be described in a subsequent report.

Acknowledgement

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References

Isaacs, A. and Lindenmann, J., Virus interference. I. The interferon. Proc. Roy. Soc. B, 147 (1957) 258.

Lockart, R.Z., Jr., Criteria for acceptance of a viral inhibitor as an interferon and a general description of the biological properties of known interferons. In N.B. Finter (Ed.), Interferons and Interferon Inducers, North-Holland, Amsterdam, 1973, p. 11.

Naito, S.-I., Tanaka, H., Tanaka, S. and Oshima, K., Kallikrein concentrations in the plasma, lymph, thoracic and cerebrospinal fluid of rabbits. Arch. Int. Pharmacodyn. Ther., 252 (1981) 162–169.