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Spectrofluorometric Determination of FUT-175 (Nafamstat Mesilate) in Blood Based on Trypsin-Inhibitory Activity

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FUT-175 (nafamstat mesilate) is a novel synthetic protease-inhibiting agent which has potent, reversible and selective inhibitory activity against trypsin-like serine proteases. A determination method for FUT-175 in biological materials, particularly in blood, was established based on the potent trypsin-inhibitory activity of this drug. The procedure consists of two steps: treatment of blood specimens with formic acid-containing ethanol for deproteinization and extraction of FUT-175 with minimal hydrolysis, and a clean-up with *n*-pentane for removal of remaining blood constituents such as phospholipids, fats and any fluorescent materials. This method shows good reproducibility and sensitivity; the detection limit was as low as 1 ng of FUT-175/ml blood.

Keywords——nafamstat mesilate (FUT-175); protease-inhibiting agent; spectrofluorometry; determination basing trypsin inhibition; formic acid-containing ethanol; *n*-pentane treatment; FUT-175 rabbit blood level

FUT-175, 6-amidino-2-naphthyl *p*-guanidinobenzoate dimethanesulfonate (nafamstat mesilate), is a novel synthetic protease-inhibiting agent developed by Torii & Co., Ltd., and its basic biochemical and pharmacological properties have already been reported by Fujii *et al.*[1,2] and Aoyama *et al.*[3] FUT-175 has been shown to inhibit the enzyme activity of trypsin-like serine proteases in a potent, selective and reversible manner, the IC_{50} value for trypsin being of the order of 10^{-8} M.[1,3]

In connection with the determination of the concentration of unchanged, biologically active FUT-175 in biological materials, particularly in blood, it was expected to be fairly difficult to produce anti-FUT-175 antibodies for use in radioimmunoassay because of the readily hydrolyzable ester linkage in the molecule. Further, separation of FUT-175 from blood constituents by high performance liquid chromatography (HPLC) with organic solvents was expected to be difficult because of its hydrophilic character.

We therefore attempted to establish a determination method based on its strong trypsin inhibitory activity. Some reports have appeared on the determination of blood concentration of drugs by methods based on enzyme inhibitory activity,[4,5] but in these studies the blood specimens were used without any pretreatment such as separative extraction or clean-up.

It is well known however that the blood contains a variety of proteolytic enzymes and their inhibitors, which might affect the results of determination if they are present in reaction mixtures. Thus, we thought it preferable to remove them by pretreatment of the blood specimens.

The method we have established consists in principle of two procedures, one being a treatment of the blood specimens with formic acid-containing ethanol to achieve simultaneous deproteinization and extraction of FUT-175 and the other being a clean-up with *n*-

pentane to remove remaining blood constituents such as phospholipids, fats and any fluorescent materials. Formic acid-containing ethanol was used to keep the pH acidic in order to minimize the hydrolysis of FUT-175 during processing.

Figure 1 shows a calibration curve obtained by the method described in Experimental, using 2 ml of chilled rabbit blood specimens to which 20 μl of 5% glucose-containing 0.0001 N HCl with FUT-175 at various concentrations (0.5—25 μg/ml) had been added. As shown in Table I and Fig. 1, this method was found to be reproducible in terms of assay results and to have a detection limit as low as 1 ng/ml blood. Recovery of FUT-175 in this method, studied by adding known amounts to blood specimens before and after extraction and clean-up procedures, was found to be dependent on the FUT-175 concentration, *i.e.* from 25.3% at 1.0 ng/ml to 45.9% at 25 ng/ml ($n=3$).

Amidinonaphthol (AN) and *p*-guanidinobenzoic acid (PGBA) are the hydrolysis products and also major metabolites of FUT-175. Among them, AN is a fluorescent compound, but its fluorescence intensity is only approx. 1/500 times that of 7-amino-4-methylcoumarin, the indicator fluorescent compound used in this method. As AN and PGBA were likely to be contained in both the blood specimens and the samples for trypsin-inhibition assay, their possible effects on the results of determination were evaluated; the assay results for FUT-175

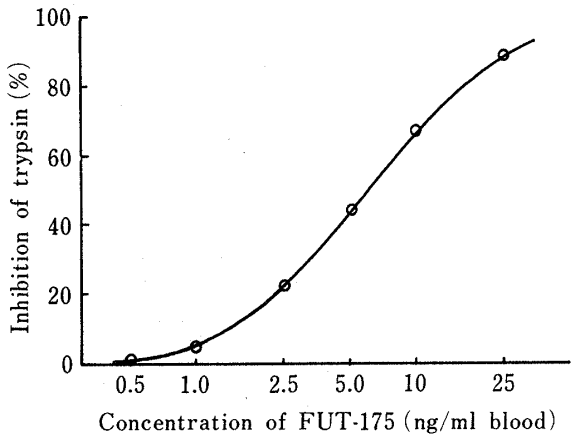

Fig. 1. Calibration Curve in the Determination of FUT-175

The blood specimens were treated as described in Experimental and 0.4 ml aliquots were applied. Each point represents the mean of 3 determinations (Table I).

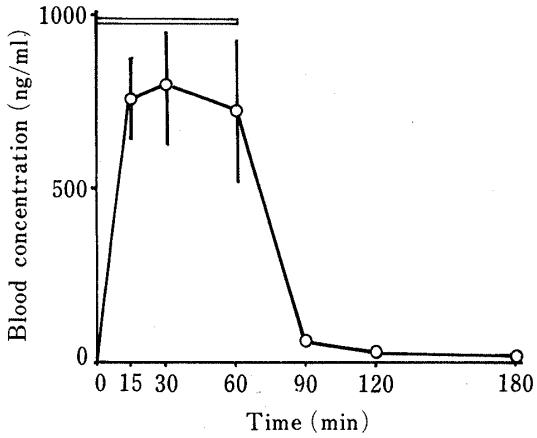

Fig. 2. Blood Level of FUT-175 in Rabbits during Intravenous Infusion at a Rate of 50 μg/kg/min and after Cessation of Infusion

Each points represents the mean of 4 rabbits and the vertical bar shows the standard error. A horizontal bar indicates the period of FUT-175 infusion.

TABLE I. Reproducibility of the Assay Method. Results of Calibration Curve Determination in Triplicate

Blood specimen No.	FUT-175 (ng/ml)					
	0.5	1.0	2.5	5.0	10.0	25.0
No. 1	1.91[a)]	5.55	20.71	44.55	65.11	89.01
No. 2	1.32	3.74	21.23	43.71	65.36	87.92
No. 3	1.61	5.15	25.35	44.92	69.51	88.32
Mean	1.61	4.81	22.43	44.39	66.66	88.42
(S.D.)	(0.30)	(0.95)	(2.54)	(0.62)	(2.47)	(0.55)
C.V. (%)	18.32	19.77	11.33	1.40	3.71	0.62

a) Each figure is the percent inhibition of trypsin activity in one determination.

(0.5 ng/ml assay mixture) were found to be the same in the absence or presence of 5 or 50 ng of either AN or PGBA, or both.

Application of this method to determine the blood level of FUT-175 in rabbits was carried out as follows: FUT-175 was infused into each of four rabbits *via* the marginal ear vein at a rate of 50 μg/kg/min for 60 min and blood specimens were taken immediately before infusion and at 15, 30, 60, 90, 120 and 180 min later. The results, shown in Fig. 2, demonstrate that the blood levels of FUT-175 were practically constant at approx. 750 ng/ml during infusion and decreased rapidly with a $T_{1/2}$ of approx. 8 min after cessation of the infusion.

In conclusion, we have established a spectrofluorometric method for the determination of FUT-175, which is a readily hydrolyzable ester compound, based on its potent trypsin-inhibitory activity, by applying a deproteinization and extraction procedure with formic acid-containing ethanol and a clean-up procedure with *n*-pentane. The method showed good reproducibility and sensitivity; the detection limit was as low as 1 ng/ml.

Experimental

Materials——Nafamstat mesilate (FUT-175), 6-amidino-2-naphthol (AN) and *p*-guanidinobenzoic acid (PGBA) were prepared at the Research Labs., Torii & Co., Ltd. Trypsin (bovine pancreas) was purchased from Sigma Chemicals. *tert*-Butyloxycarbonyl-L-phenylalanyl-L-seryl-L-arginine 4-methylcoumarinamide (Boc–Phe–Ser–Arg–MCA) and 7-amino-4-methylcoumarin (AMC) were purchased from the Peptide Research Foundation, Osaka. Other chemicals used were purchased from Wako Pure Chemicals.

Formic acid-containing ethanol was ethanol containing aq. 1/2 N formic acid at a concentration of 1 percent. Tris–HCl buffer was a mixture of 1/2 M Tris and 1/2 N HCl, pH 7.4, containing 2.5 mM $CaCl_2$. The substrate, Boc–Phe–Ser–Arg–MCA, was dissolved in *N*,*N*-dimethylformamide (fluorometric grade) at a concentration of 5 mg/ml. Trypsin was dissolved in Tris–HCl buffer at a concentration of 10 ng/ml.

Apparatus——Fluorescence was measured with a Hitachi MPF-3 fluorescence spectrophotometer.

Assay Procedure——a) Preparation of Samples for Trypsin-Inhibition Assay: A blood specimen (2 ml), immediately after being drawn, was added to chilled formic acid-containing ethanol (20 ml) and the mixture was shaken for 15 min, followed by centrifugation at 3000 rpm for 15 min. The supernatant (18 ml) was evaporated to dryness *in vacuo*. The residue was dissolved in 0.0001 N HCl (1 ml). The solution was well mixed with a mixture of *n*-pentane (5 ml) and ethanol (0.5 ml), shaken for 10 min and centrifuged at 3000 rpm for 15 min. The organic layer was removed by aspiration, and the residual aqueous layer was shaken with *n*-pentane for 10 min, followed by centrifugation at 3000 rpm for 15 min. The same procedures for clean-up with *n*-pentane were repeated 3 times. Formic acid-containing ethanol (4 ml) was added to the finally obtained aqueous layer and the mixture was evaporated to dryness *in vacuo*. The residue was dissolved in 0.0001 N HCl (2 ml) and the solution thus obtained was used as the sample for trypsin-inhibition assay.

b) Trypsin-Inhibition Assay: Tris–HCl buffer (1.4 ml) and trypsin solution (0.2 ml) were added to a sample solution (0.4 ml). The mixture was incubated for 3 min at 37 °C. After addition of the substrate solution (20 μl), the mixture was incubated for 30 min at 37 °C, followed by addition of 50% acetic acid (0.2 ml) to stop the reaction. The fluorescence of AMC liberated was measured with a fluorescence spectrophotometer (Ex 380 nm and Em 440 nm) against a reagent blank. As a reference, 100% trypsin activity was determined using Tris–HCl buffer (0.4 ml) instead of a sample solution (0.4 ml) in the starting reaction mixture.

The concentration of FUT-175 in the blood specimen was calculated from the calibration curve.

Calibration Curve——A calibration curve was obtained by the method described above using 2 ml aliquots of chilled blood specimens to which 20 μl portions of 5% glucose-containing 0.0001 N HCl with FUT-175 at various concentrations (0.5—25 μg/ml) had been added.

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