

## Studies on Oral Fibrinolytic Therapy : Application of High Molecular Weight Form Urokinase for Experimental Thrombus in Beagle

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### SUMMARY

Enteric coated capsule of urokinase which contains highly purified high molecular weight form urokinase (mol. wt. 53,000 ; S.A. 103,000 IU/mg protein), enzyme stabilizing factors and the accelerators of intestinal absorption was prepared. In the present study, it was confirmed using beagle that urokinase orally administrated could be absorbed from intestine into blood and activate the endogenous fibrinolytic enzyme system. It was further confirmed that when compared with urokinase intravenously administrated, oral urokinase could more effectively activate the endogenous fibrinolytic enzyme system and its efficacy also continued for longer interval after administration.

### INTRODUCTION

Since Tillet and Garner<sup>1)</sup> discovered streptokinase from beta hemolytic streptococci and Ratnoff<sup>2)</sup> firstly demonstrated that streptokinase (SK) reacted stoichiometrically with human plasminogen, SK has been clinically used as a thrombolytic agent by many workers.<sup>3-8)</sup> However, the antigenicity of SK in human caused the halt to development of clinical use of this agent and made necessary the discoveries of another new thrombolytic agent. Urokinase(UK), a plasminogen activator purified from human urine and lacking antigenicity in human,<sup>9-11)</sup> was a reasonable choice. Several

studies have indicated UK to be a potent thrombolytic agent capable of blood clot dissolution.<sup>12-14)</sup> In spite of many accumulation of clinical trails of UK as a thrombolytic agent, the adequate dose and method of administration to bring about maximum effect become still unclear. It is well known that UK has a few but no affordable disadvantage : After the intravenous injection of UK is rapidly excreted and also inactivated by several plasmin inhibitors such as  $\alpha_2$ -macroglobulin,<sup>18)</sup>  $\alpha_2$ -plasmin inhibitor,<sup>19)</sup>  $\alpha_1$ -antitrypsin,<sup>20)</sup> antithrombin III and PAI-1,<sup>21)</sup> so that its duration of activity is short, necessitating continuous infusion of very high doses, which are prohibitory expensive and also painful for patients. For instance, Fletcher<sup>15)</sup> used a loading dose of 250,000 CTA units over 10 min followed by a maintenance dose of 230,000-500,000 CTA units per hr. More recently, Samama et al.<sup>23)</sup> administrated UK to 45 patients as a continuous infusion for approximately 15 hr. The initial loading dose of 150,000 CTA units was followed by either 1,000, 2,000 or 2,500 CTA units/kg body weight per hr. They concluded that 2,500 CTA units/kg body weight per hr consistently induced a thrombolytic state. On the contrary, in 1976 Bell<sup>24)</sup> indicated that there was no evidence of greater clot dissolution in patients with who received high dose of UK, than those who received low dose. The authors also proposed the possibility that the use of an excessive

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amounts of UK in fibrinolytic therapy might interfere with the appearance of fibrinolytic activity in plasma of patients.<sup>25)</sup> These facts raise the possibility that the best method of administration of UK to bring about maximum efficacy in fibrinolytic therapy might be the continuous and gradual infusion optimal amounts of UK into blood. As a matter of fact, administration method like this by intravenous injection is, however, almost impossible, because of patient's agony and lability of UK during infusion procedure. Therefore, in the present study, oral administration method of UK was experimentally examined using beagle.

## MATERIALS AND METHODS

### Chemical reagents

Chromogenic substrate S-2444 (Kabi Diagnostica, Sweden), bovine thrombin (Mochida Pharmaceutical Co., Ltd., Japan), canine fibrinogen type V (Sigma, U.S.A).

### Preparation of UK

Urine origin UK was highly purified by using affinity chromatography. Five hundred mg of partially purified urokinase (Towa Kako. Co., Ltd., Tokyo; SA 11,000 IU/mg protein) was dissolved in 150 ml of 0.1M phosphate buffer containing 2 M NaCl, pH 7.4 and applied on [N $\alpha$ -( $\epsilon$ -aminocaproyl)-DL-homoarginine hexylester]-Sephadex column (2.3 $\times$ 13 cm) equilibrated with the same buffer. After washing UK adsorbed was eluted with 0.1 M phosphate buffer containing 2 M NaCl and 6 M urea, pH 7.4. This was further purified by gel filtration on Sephadex G-100, equilibrated with 0.15 M ammonium bicarbonate. The main peak of UK detected by S-2444 amidolysis was collected and lyophilized. The final preparation showed a single protein band with mol.wt. 53,000 on SDS-acrylamide gel electrophoresis and the mol.wt. of this UK was not altered by incubation at 37°C for 12hr in neutral pH buffer. The specific activity (S.A.) was 103,000 IU/mg protein, approxi-

mately agreeing with those reported for crystalline UK (104,000 CTA U/mg protein) by Lesuk et al.,<sup>26)</sup> and type S<sub>2</sub> human UK (93,000 CTA U/mg protein) by White et al.<sup>27)</sup> By p-nitrophenylguanidinobenzoate titration,<sup>28)</sup> 92.8% of this enzyme was active.

### Preparation of UK capsule

Thirty thousand IU of highly purified UK was added 45 mg of bovine serum albumin (Merck, West Germany), 150 mg of swine gastric mucin (Yamanouchi Pharmaceutical Co., Ltd., Japan), 50 mg of mannitol (Towa Kasei Co., Ltd., Japan), 45 mg of microcrystalline cellulose (Asahi Chemical Indust. Co., Ltd., Japan) and 3 mg of magnesium stearate (Taihei Chemical Indust. Co., Ltd., Japan), and packed in gelatin capsule which enterically coated. The placebo capsule contained above components without UK and was enterically coated.

### Experimental thrombus in dog's saphenous vein

For experimental animal, beagle (8-10kg body weight, female) which operated artificial thrombus was used. After external saphenous vein dissected from surrounding tissue, and all of branches were ligated, the vein running along tibia was clamped for 3 cm intervals. Then, full blood in the vein was removed by saline solution, and experimental thrombus was formed with 0.4 ml of canine fibrinogen (5%) and 0.2 ml of bovine thrombin (50 U/ml). The formation of artificial thrombus was confirmed by X-ogram, and this experimental thrombus did not lyzed till 24 hr.

### Administration method of UK capsule

Oral administration group; UK capsule (containing 30,000 IU) was orally administered to 6 beagles. Intravenous administration group; highly purified UK preparation (30,000 IU) was intravenously injected to 6 beagles by one shot method. Control group; capsule without UK was given to 6 beagles. Venous blood samples were collected at 0.5, 1, 2, 3, 6, 9, 12 and 24 hr after administration.

In case of oral administration group, the reaching of UK capsule into duodenum which was found by X-ographical method, was defined as the initial point.

#### Collection of blood

Blood was collected from median cephalic vein by the plastic syringe. One volumes of 3.8 % sodium citrate was added immediately to nine volumes of the blood. Plasma was obtained by centrifuging the mixture at  $1580 \times g$  for 10 min at  $4^{\circ}C$ .

#### Measurement of UK activity

Amidolytic activity of UK was measured by the method of Claeson et al.<sup>29)</sup> Plasma euglobulin lysis time (ELT) was determined ; the plasma euglobuline was precipitated from 0.5 ml plasma according to the method of Milston (19 volumes water containing 1.32 volume of 1 % acetic acid added to 1 volume plasma).<sup>30)</sup> After centrifugation, the precipitate was resuspended in 0.5 ml of 0.01 M Veronal buffer, pH 7.4 and clotted with 0.5 ml of thrombin (50 U/ml). Clots were incubated at  $37^{\circ}C$  and the time for complete lysis was recorded by Clots Lysis Time Recorder (Riko Shoji Co., Ltd., Japan). Plasma fibrin/fibrinogen degradation products (FDP) was measured by latex-aggregation method with canine FDPL-kit (Zeria Pharmaceutical Co., Ltd., Itabashi Lab., Japan). The test sensitivity for canine fibrinogen was found to be  $1 \mu g/ml$ .

#### Thromboelastgraphy (TEG)

Thromboelastgraphy originated by hartert was performed by TEG apparatus (Hellige) according to the routine procedure. The instrument used advances the film with a speed of 1 mm per min.

#### Assay of plasma coagulation factor

Coagulation system were measured by activated partial thromboplastin time<sup>31)</sup> and recalcification time with Clot Digitim TE20 (Erma Optical Works Co., Ltd., Japan).<sup>32)</sup>

#### Macroangiography of artificial thrombus

Macroangiography was performed by the method of Bergstrom et al.<sup>33)</sup> The cannulation

for macroangiography was distributed from femoral artery which located in the upper portion of artificial thrombus, and X-ray photograph was taken at each 3 hr after thrombus formation.

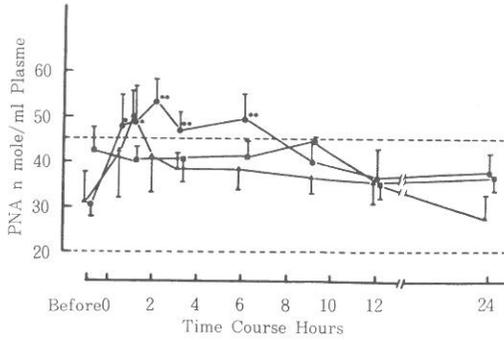
## RESULTS

#### UK activity in plasma

In the present study, the significant differences of amidolytic activity, ELT and FDP level in plasma or serum were examined between before and after administration of UK. The physiological range was calculated from the all data obtained before treatment and defined as mean  $\pm$  standard error. The UK activity in plasma of 3 experimental groups were measured by use of the specific chromogenic substrate S-2444. As shown in Fig 1, in oral administration group, the significant UK activity appeared at 30 min after reaching of UK capsule to duodenum (mean  $\pm$  SE =  $48.2 \pm 8.1$  nmol /ml plasma ;  $p \leq 0.05$ ), gradually increased and reached to a maximum at 2 hr (1 hr ;  $49.0 \pm 8.4$  ;  $p \leq 0.05$ , 2 hr ;  $53.1 \pm 4.9$  ;  $p \leq 0.01$ ), after which further continued from 3 hr to 6 hr (3 hr ;  $47.7 \pm 4.3$  ;  $p \leq 0.01$ ; 6 hr ;  $49.8 \pm 5.8$  ;  $p \leq 0.01$ ). On the other hand, in intravenous administration group, the UK activity attained a maximum which was without significant difference at 1 hr ( $50.5 \pm 6.8$ ), and then returned to physiological range at 2 hr. In control group, the UK activities at all intervals remained in the physiological range. From these results, it was supposed the possibility that UK orally administered might be gradually absorbed from duodenum or intestine into blood and that absorbed UK might be remained in blood for longer intervals when compared with results of intravenously administrated UK.

#### Effect of oral UK on blood fibrinolysis

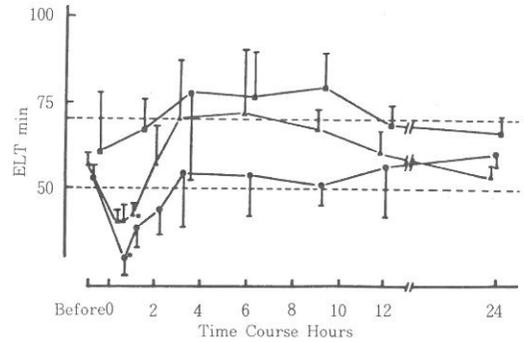
The ELT was measured by Fibrin Clots Lysis Time Recorder and FDP was measured by using the anti-coated latex particles. As shown in Fig. 2, the significant shortening



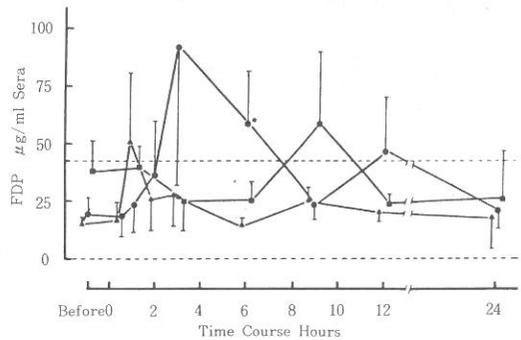
**Fig.1 Amidolytic activity of urokinase**  
 The amidolytic activity of UK was measured with 0.1ml of 3mM S-2444 solution mixed 0.2 ml of plasma. The activity was represented as nanomole of pNA revealed by S-2444.  
 ●—● ; Oral administration group (30,000 IU/body)  
 ▲—▲ ; Intravenous administration group (30,000 IU/body)  
 ■—■ ; Control group  
 \* ;  $p \leq 0.05$ , \*\* ;  $p \leq 0.01$  : Significant difference from the physiological range, mean  $\pm$  SE =  $32.5 \pm 12.5$

of ELT was recognized at 30 min ( $30.2 \pm 3.6$ ;  $p \leq 0.05$ ) in oral administration group, whereas at 1 hr ( $41.4 \pm 2.8$ ;  $p \leq 0.05$ ) in intravenous administration group. The ELT in both group returned to physiological range at 3 hr. Any significant difference was not statistically recognized between the ELT time course pattern in oral administration group and that in intravenous administration group. On the contrary, there was no significant shortening of ELT in control group. Fig. 3 shows the results of serum FDP level. In control group, FDP level slightly increased at 9 hr, but the level showed without significant difference. In intravenously administration group, it reached to a maximum at 1 hr, after which was followed by rapid return to a physiological range. The FDP level at 1 hr also showed without significant difference. On the contrary to these results, in oral administration group, FDP level remarkably increased at 6 hr ( $59.0 \pm 23.8$ ;  $p \leq 0.01$ ). From all these results, it was confirmed that oral UK which reached into circulating blood succeeded in activating the fibrinolytic enzyme system. It was also confirmed that as well as the results of the

UK activity, the significant blood fibrinolytic activity when orally administered was recognized for obviously longer intervals after administration than that when intravenously administered.



**Fig.2 Euglobulin clots lysis time (ELT)**  
 Euglobulin fraction was dissolved with 0.5ml of 0.01mM Veronal buffer containing 0.1M NaCl (pH 7.4) and immediately added to 0.1ml of 50 U/ml thrombin solution. ELT was recorded by Fibrin Clots Lysis Time Recorder.  
 ●—● ; Oral administration group (30,000 IU/body)  
 ▲—▲ ; Intravenous administration group (30,000 IU/body)  
 ■—■ ; Control group  
 \* ;  $p \leq 0.05$ , \*\* ;  $p \leq 0.01$  : Significant difference from the physiological range, mean  $\pm$  SE =  $60 \pm 10$



**Fig.3 The measurement of FDP**  
 The serum FDP level was measured by FDPL-kit using 0.01ml of serum or its dilution.  
 ●—● ; Oral administration group (30,000 IU/body)  
 ▲—▲ ; Intravenous administration group (30,000 IU/body)  
 ■—■ ; Control group  
 \* ;  $p \leq 0.05$ , \*\* ;  $p \leq 0.01$  : Significant difference from the physiological range, mean  $\pm$  SE =  $21 \pm 21$

**TEG patterns after oral administration**

TEG patterns in intravenous administration group and oral administration group are shown in Fig. 4. In intravenous administration group,

any fibrinolytic patterns in TEG after administration was not recognized, while oral administration group showed the obvious fibrinolytic patterns at 1 hr and 2 hr after administration. However, any hypo or hypercoagulability patterns were not recognized in TEG.

To examine the effect of orally or intravenously administrated UK for blood coagulation factor, the APTT and Recalcification time were respectively measured. As shown in Fig. 5, both APTT and Recalcification time did not indicate significant differences among control, intravenous and oral administration groups, and all titres remained in physiological range.

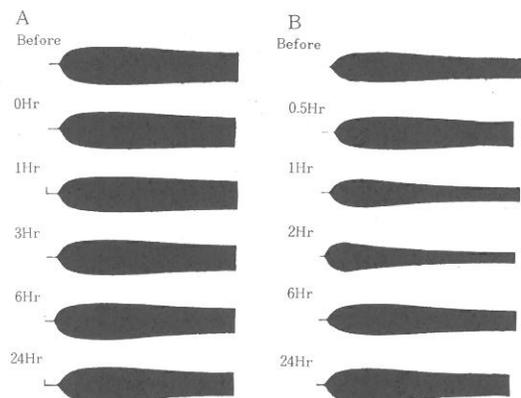


Fig.4 Thromboelastography

TEG patterns in intravenous administration group (A) and in oral administration group (B) were recorded with employing 0.4ml of blood by TE-40 Clot Tracer.

#### Macroangiography of artificial thrombus of external saphenous vein

In the present study, to finally confirm the effect of orally administrated UK for blood fibrinolytic enzyme system, the lysis of artificial thrombus was investigated by macroangiography. As summarized in Table I, in control group, the artificial thrombus spontaneously dissolved at 18-24 hr after thrombus formation and blood stream repressed. In oral administration group, the lysis of artificial thrombus was confirmed at 6-9 hr after administration, which results were almost in accord with those in intravenous administra-

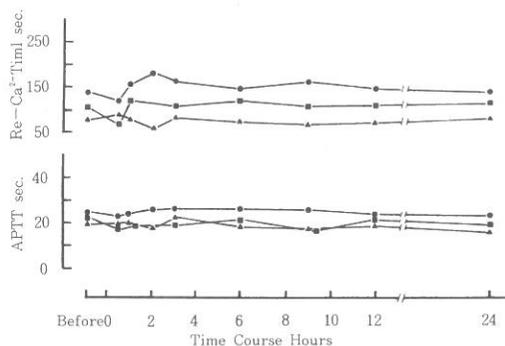


Fig.5 Activated partial thromboplastin time (APTT) and recalcification time (Re-Ca<sup>++</sup> Time)

APTT ; 0.1ml of plasma was mixed with 0.1ml of APTT reagent and the mixture was incubated for 4 min at 37°C. 0.1ml of 0.025M CaCl<sub>2</sub> was added and clotting time was measured by TE-20.

Re-Ca<sup>++</sup> Time ; 0.1ml of plasma was mixed with 0.2ml of 0.025M CaCl<sub>2</sub>. Clotting time was measured by the same method.

●—● ; Oral administration group (30,000 IU/body)  
 ▲—▲ ; Intravenous administration group (30,000 IU/body)  
 ■—■ ; Control group

tion group (3-6 hr).

#### DISCUSSION

The proteolytic drugs have been clinically used by intravenous, intramuscular, intracutaneous injection, buccal and oral administration. Especially, streptokinase, trypsin,  $\alpha$ -chymotrypsin, lysozyme, papain, bromeline and etc. have been orally administrated. The administration method of UK except intravenous administration method, however, had yet been developed. Now, another administration method of UK than intravenous injection has been required for the efficient utilization of UK, because intravenous administration method of UK possesses some disadvantages as described above. In the present study, therefore, the efficacy of UK orally administrated was investigated by using beagle and was further compared with the efficacy of UK intravenously administrated.

As well known, two forms of UK have been clinically used. One is that seems to represent of native UK with molecular weights of about 54,000 and specific activity (CTA

**Table I : Thrombolytic observation by macroangiography**

An artificial thrombus was prepared in the saphenous vein of beagles by removing the blood from a section of the vein and adding 0.4ml of 5% canine fibrinogen and 0.2ml of bovine thrombin (50 U/ml). The formation of the artificial thrombus was confirmed by angiography. X-ray photographs were taken after infusion of 2ml of angiographin during 0.1sec ; + recanalization, - without recanalization.

Case.No.	hr	Thrombolytic action after thrombus formation						
		0	3	6	9	12	18	24
1		-	-	+	+	+	+	+
2		-	+	+	+	+	+	+
3		-	+	+	+	+	+	+
4		-	+	+	+	+	+	+
5		-	-	+	+	+	+	+
6		-	+	+	+	+	+	+
7		-	-	+	+	+	+	+
8		-	-	+	+	+	+	+
9		-	-	-	+	+	+	+
10		-	-	+	+	+	+	+
11		-	-	-	+	+	+	+
12		-	-	+	+	+	+	+
13		-	-	-	-	-	-	+
14		-	-	-	-	-	+	+
15		-	-	-	-	-	-	+
16		-	-	-	-	-	-	+
17		-	-	-	-	-	-	+
18		-	-	-	-	-	+	+

units/mg) of 100,000, the other is that seems to represent the enzymatic degradative UK during purification from urine (mol.wt. 33,000, S.A. 220,000). On the other hand, purification of other UK from tissues or cell cultures has also been reported.<sup>36)</sup> In spite of many reports on these various types of UK, it has still been unclarified which type of UK could be most effectively utilized for venous and arterial thrombotic diseases. Recently, it has also been reported that native type of commercial UK preparations was contaminated with some coagulation factors which might induce the important interference in fibrinolytic therapy. In the present study, therefore, to prepare the UK capsule, native UK was firstly purified by the method described in the text, and then intermingled with bovine serum albumin and swine mucin as stabilizer or promoter of absorptiveness.

In order to confirm whether or not purified UK in oral capsule could be absorbed from intestinal membrane into blood, the amidolytic activity on chromogenic substrate in plasma

was determined at each interval after administration of UK. As shown in Fig. 1, the significant elevated amidolytic activity was determined from 30 min to 6 hr after oral administration of UK. As the amidolytic activity can be also detected in non-treated plasma, it is assumed that the detection of amidolytic activity may be due to other activators or components than UK. However, it is obvious that the significant elevated amidolytic activity from physiological range after oral administration of UK are due to UK which absorbed into blood, and it is also

supposed the possibility that UK orally administered may be very gradually absorbed from intestine into blood. On the other hand, when UK intravenously administered, the amidolytic activity in plasma did not show any significant difference from physiological range at all intervals after administration. It is not clear, however, why on the contrary to the results of UK orally administered, the significant difference of amidolytic can't be detected when UK was intravenously injected. There is the possibility that large amounts of UK intravenously injected by one shot might be combined with some protease inhibitors, hence rapidly inactivated, while gradual absorption of small amounts of UK from intestine into blood might bring about a conformational change of UK, which consequently protects UK from binding with some protease inhibitors. However, this supposition is also still open to speculation, because in the present study, it is still unclear why or how can be absorbed from intestine into blood.

Next, in order to confirm whether or not

UK absorbed into blood can activate the endogenous fibrinolytic system, ELT, FDP and TEG were investigated in plasma, serum or whole blood of experimental animals. As shown in Fig. 2 and 3, UK orally administered succeeded in activating the fibrinolytic enzyme system. It was particularly interesting that as well as the results of amidolytic activity, the significant shortening of ELT and the significant titres of FDP were more prominently recognized and continued for longer intervals after administration than those when UK intravenously injected. These results are supposed to be a phenomenon which followed by gradual activation of endogenous fibrinolytic enzyme system. As shown in Fig. 4, TEG in oral administration group also more clearly indicated the fibrinolytic pattern than that in intravenous administration group.

In the present study, to finally confirm the efficacy of oral UK in thrombolytic therapy, the lytic action of oral UK was examined for artificial thrombus of beagle. As shown in Table 1, UK which was orally administered and absorbed into blood made possible to dissolve the artificial external saphenous thrombus of beagle.

It has been stated that a great deal of UK must be intravenously injected during a long

intervals, in spite of patient's agony and prohibitory expense. From all experimental results in the present study, the authors proposed the possibility that if UK is orally administered for patients with thrombotic disease, UK will be able to be more effectively utilized without patient's agony and prohibitory expense, and the authors also strongly believe that oral administration method of UK will maybe bring about a rapid progress in the fields of thrombolytic therapy and chemotherapy.

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