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REMOVAL OF THE ANTICOAGULANT ACTIVITIES OF THE LOW MOLECULAR WEIGHT HEPARIN FRACTIONS AND FRAGMENTS WITH FLAVOBACTERIAL HEPARINASE

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ABSTRACT Recently, the development of low molecular weight heparin fractions and fragments (LMHF) as potential antithrombotic agents has gained increased attention. However, the lack of antagonists to neutralize the anticoagulant effects of these drugs may seriously exclude them from possible uses in extracorporeal therapy. This is mainly because of the concern that the high dosage of the drugs employed in extracorporeal therapy could lead to serious bleeding risks. Our earlier work has demonstrated that immobilized heparinase can remove polydisperse heparin both *in vitro* and *in vivo*. To examine whether such a system may be used as a novel approach to neutralize the anticoagulant effects of LMHF, different LMHF were tested using heparinase. *In vitro* data showed that both the APTT and anti-FXa activities of the LMHF including Kabi 2165, PK 10169, CY 216 and CY 222 were nearly completely eliminated by heparinase in less than 20 min. This study suggests that an immobilized heparinase system may be an useful element for the acceptance of the LMHF for their use in extracorporeal therapy.

Key words: Heparinase, low molecular weight heparin fractions and fragments, neutralization, APTT activity, anti-FXa activity.

INTRODUCTION

Heparin inhibits the proteases of blood coagulation and is therefore used universally as an effective antithrombotic agent for the treatment and prophylaxis of many thromboembolic disorders, for maintaining the fluidity of blood in extracorporeal circulation, and in surgery of the heart and blood vessels (1). However, its clinical use is sometimes associated with hemorrhagic complications (2,3). It is assumed that the antithrombotic effects of heparin are associated with heparin's binding to antithrombin III and the consequent effects of this complex on anti-FXa activity, and the hemorrhagic effects are associated with heparin's potency in prolonging the activated partial thromboplastin time (APTT) (1). Recently, the dichotomy between the anti-FXa activity and the antithrombin activity of heparin has been found to be dependent on heparin's molecular weight (4,5), leading to the development of the low molecular weight heparin fractions and fragments (LMHF). In numerous experimental models, these LMHF have been shown to markedly activate the anti-FXa activity, yet only slightly prolong the APTT, and therefore lessened the tendency of induced hemorrhage (6-9). However, owing to the small molecular size and weight for the LMHF, protamine, a conventional antagonist to heparin, can no longer bind with these compounds effectively and neutralize their anticoagulant functions (10-12). For certain clinical situations such as kidney dialysis and open heart surgery where a high dose of anticoagulant is required, bleeding risks may be higher with the employment of the LMHF than that of heparin, simply because of the lack of an available and appropriate neutralizing agent.

In earlier work, we demonstrated that an immobilized heparinase system can be placed at the termination of an extracorporeal device to remove the anticoagulant effects of the high level of heparin encountered during extracorporeal therapy (13). Heparinase degrades polydisperse heparin to inactive, chain-shortened fragments with an average molecular weight of about 1,000 daltons (14). Since the LMHF currently available have relatively higher molecular weights (2,500 - 6,000 daltons), it was hypothesized that heparinase may be capable of degrading these LMHF as well. To test this possibility, different LMHF including Kabi 2165, Pharmuca PK 10169, Choay CY 216 and CY 222 were tested for their reaction with heparinase. Removal of anticoagulant activities (antithrombin and anti-FXa) of these LMHF by heparinase was also examined in plasma. Success in this approach to neutralization by the immobilized heparinase system may be useful to the acceptance of LMHF for their clinical uses in extracorporeal therapy.

MATERIALS

Heparin, sodium salt from porcine intestinal mucosa, was purchased from Hepar (Franklin, Ohio). Kabi 2165 was a gift from Dr. Holmer and Dr. Andersson at KabiVitrum AB, Stockholm. PK 10169 was generously supplied by Dr. Mardiguian at the Pharmuca Laboratories, Gennevilliers, France. CY 216 and CY 222 were kindly provided by Dr. Choay at the Institute of Choay, Paris, France. The mean molecular weights, specific activities in anti-FXa units and APTT units per mg and relative ratios of anti-FXa

activity over APTT activity for heparin and each of the LMHF are listed in Table I.

TABLE I

Properties of Heparin and LMHF

	Supplier	Mean Molecular Weight	Anti-FXa Units/mg	APTT Units/ mg	Anti-FXa: APTT Ratio
Heparin	Hepar	13,500	150	150	1.0
Kabi 2165	Kabi	5,500	155	40	3.9
PK 10169	Pharmuca	4,500	130	42	3.1
CY 216	Choay	4,500	200	29	6.9
CY 222	Choay	2,500	250	11	22.7

Actin Activated Cephaloplastin Reagent (for APTT measurements) was from Dade Diagonostics Inc., Aguada, Puerto Rico. Substrate S-2222, antithrombin III, Factor Xa, antithrombin III, human normal plasma and buffer (0.05 M Tris, 7.5 mM EDTA, pH 8.4) for the anti-FXa measurements were obtained as a Coatest Heparin Kit from KabiVitrum, Stockholm. Protamine sulfate (grade III from Herring) was from Sigma Chemical Company, St. Louis, MO. Bio-Gel A agarose (8%) was obtained from Bio-Rad Laboratories, Richmond, California. Human blood was collected in citrate (9:1, v/v whole blood to 3.8 % weight trisodium citrate) from paid donors at Children's Hospital Medical Center Blood Bank, Boston, Massachusetts. All chemicals were reagent grade, and water was twice distilled.

METHODS

Heparinase Preparation and Immobilization

Heparinase, produced by *Flavobacterium heparinum* (15) was purified using batch hydroxylapatite chromatography (16). The partially purified heparinase was used directly or was immobilized on cross-linked 8 % agarose beads pre-activated with cyanogen bromide, according to the method described previously (17). One unit of heparinase activity was defined as the amount of enzyme which degrades 1 mg of heparin/h.

Assay for Activated Partial Thromboplastin Time (APTT)

100 µL of Activated Cephaloplastin Reagent prewarmed to 37°C were incubated with 100 µL of plasma sample at 37°C for 2 min, followed by the addition of 100 µL of 0.02 M $CaCl_2$. After another 25 sec, a platinum innoculating loop was drawn through the mixture until a clot was formed and the time was recorded. The APTT was compared to a standard curve prepared for each of the individual LMHF at concentrations of 0 - 0.6 units/mL. The APTT units were defined using the 3rd Heparin International Standard as reference. Samples for measurements were first diluted with normal human plasma until the APTT lay within the linear range of the standard curve.

Assay for Anti-FXa activity

To a test tube, 800 L of 0.2 M Tris-EDTA buffer (pH 7.4) were mixed with 100 µL antithrombin III, followed by the addition of 100 L of plasma sample. 200 µL of this mixture were incubated at 37°C and 100 µL of Factor Xa solution (7 nkat/mL) were added. After 30 seconds, 200 µL of S-2222 (0.75 mg/mL) were added. The reaction was quenched after 180 seconds by the addition of 300 µL of 50 % acetic acid. The absorbance at 405 nm was measured and compared to a standard curve prepared for each of the individual LMHF at concentrations of 0 - 0.1 units/mL. The anti-FXa units were defined using the 3rd Heparin International Standard in the Yin and Wessler test (18). Samples for measurements were first diluted with human plasma until their absorbance at 405 nm lay within the linear range of the standard curve.

Degradation of LMHF by Heparinase in Aqueous Buffer

Heparinase is an eliminase which cleaves heparin at the α-1,4 glycosidic linkage to produce α,β unsaturated uronides. The non-reducing end of the chain-shortened heparin fragments is a chromophore which possesses an absorption maximum at 232 nm (19). The degradation of LMHF by heparinase was monitored by the appearance of these ultraviolet adsorbing heparin fragments. One mL of each of the LMHF preparations (25 mg/mL) was mixed with 0.1 mL of heparinase solution (0.3 mg/mL, specific activity: 200 units per mg of protein) and incubated at 37°C. At various times, 25 µL of the reaction mixture were withdrawn and quenched in 1.5 mL of 0.03 M HCl. The time-dependent increase in absorbance at 232 nm was used to calculate the initial rate of the reaction. To determine the Michaelis-Menten rate constant (Km) of the heparinase reaction for each of the LMHF, the initial rates of degradation were measured as a function of the substrate concentration ranging from 0.1 - 25 mg/mL. The Km value was then estimated from the slope of the Lineweaver-Burk plot. The molar quantities of the degradation products generated by each milligram of the LMHF sample, as well as the average molecular weight of the degradation products was estimated from the absorbance at 232 nm in the digestion media after degradation was completed, using an extinction coefficient of 5.1×10^3 cm^{-1} M^{-1} for the α,β unsaturated uronides (19). Unless otherwise stated, the buffer contained 0.25 M sodium acetate, 2.5 mM calcium acetate at pH 7.

Neutralization of LMHF by Protamine in Human Plasma

Normal heparin (from Hepar) was prepared at a concentration of 0.01 mg/mL in human plasma, while LMHF were prepared at a concentration of 0.1 mg/mL. To nine parts of the heparinized plasma one part of the protamine solution at concentrations ranging from 0.05 to 3.0 mg/mL was added. After 5 min of incubation at 37°C the residual APTT and anti-FXa activities were measured. The residual activity was represented as the relative activity assuming a 100 % value for the initial activity.

Removal of Anticoagulant Activity of LMHF by Immobilized Heparinase

Plasma containing heparin or LMHF was prepared as described above. To 9 mL of the heparinized plasma, 5 mL of cross-linked 8 % beads (a 75 % suspension in physiologic saline solution) containing immobilized heparinase (145 units/mL of beads) were added. The mixture was incubated at 37°C with gentle agitation. At time intervals of 0, 10, 20, 40, and 60 min during the incubation, an aliquot of 1 mL of the mixture was withdrawn and centrifuged at 3,000 g, 0°C for 3 min to remove the beads and quench the reaction. The residual APTT and anti-FXa activities in the supernatant were measured. The residual activity was represented as the relative activity assuming a 100 % value for the initial activity.

RESULTS

Degradation of LMHF by Heparinase in Aqueous Buffer

Table II summarizes the results of the enzymatic degradation of heparin and LMHF with heparinase. As indicated by their different Km values, heparinase appears to exhibit completely different affinities for different LMHF, despite similar manufacturing procedures and even similar molecular weight distribution for these LMHF. For instance, while both Kabi 2165 and PK 10169 display similar molecular weights, PK 10169 shows a Km value ten times higher than that of Kabi 2165. This difference in Km values may be accounted for in terms of the different molecular compositions and/or chemical structures present in these compounds. However, almost equal molar quantities of final degradation products were produced by each gram of heparin and LMHF (with the exception of PK 10169), suggesting that the reaction mode catalyzed by heparinase might be similar for heparin and LMHF. The identity in average molecular weight of the final degradation products also reflects such a conclusion. It is not clear what causes the discrepency between the reaction kinetics and the reaction mode. One explanation would be that the initial binding sites on heparin and LMHF for heparinase binding might not be those involved in cleavage.

TABLE II

Degradation of Heparin and LMHF by Heparinase

	Average Molecular Weight	Km (M)	Moles of Degradation Products/per gram of substrate	Average Molecular Weight of Degradation Products
Heparin	13,500	0.09	0.94	1,060
Kabi 2165	5,000	0.15	0.96	1,040
PK 10169	4,500	1.00	0.81	1,410
CY 216	4,500	0.60	0.90	1,110
CY 222	2,500	0.90	0.92	1,090

Neutralization of LMHF by Protamine in Human Plasma

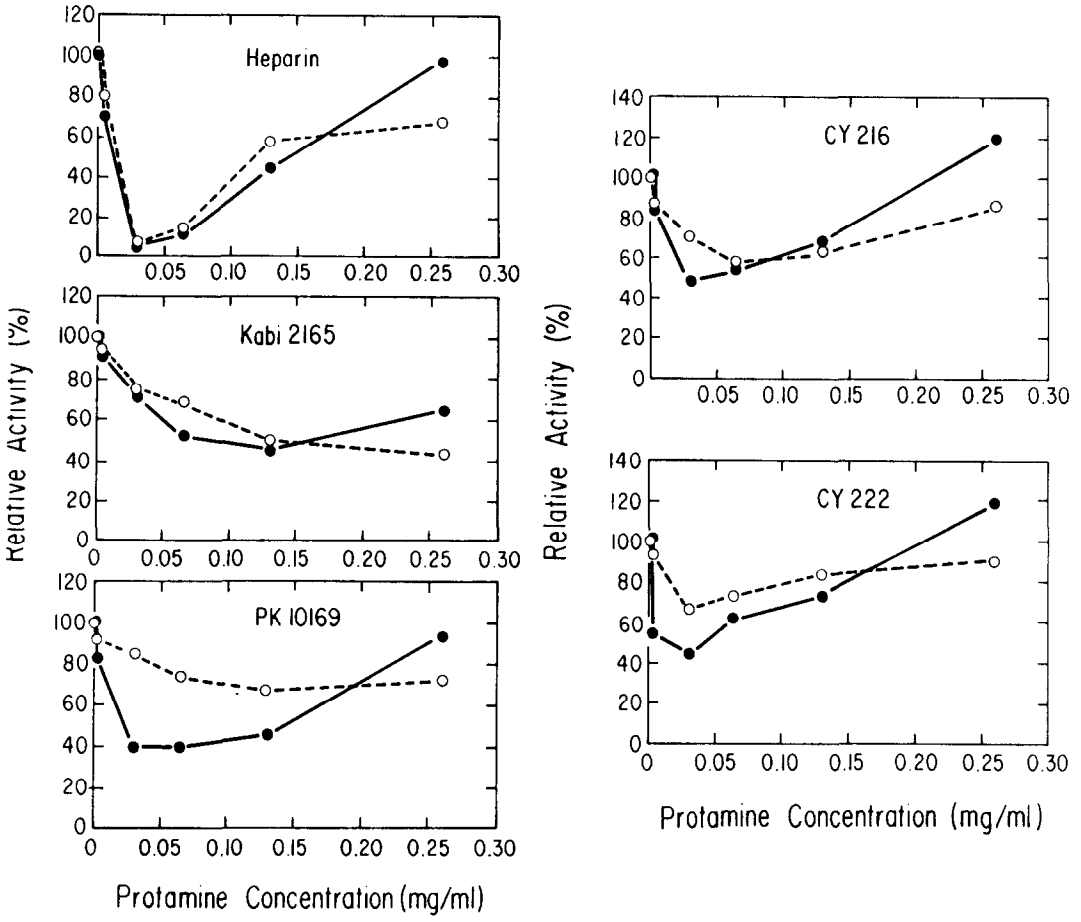

FIG. 1

Neutralization of LMHF by protamine sulfate in human plasma. ●—●—●, APTT activity. O---O---O, anti-FXa activity. The residual activity was represented as the relative activity assuming a 100 % value for the initial activity. For details, see "Methods" section.

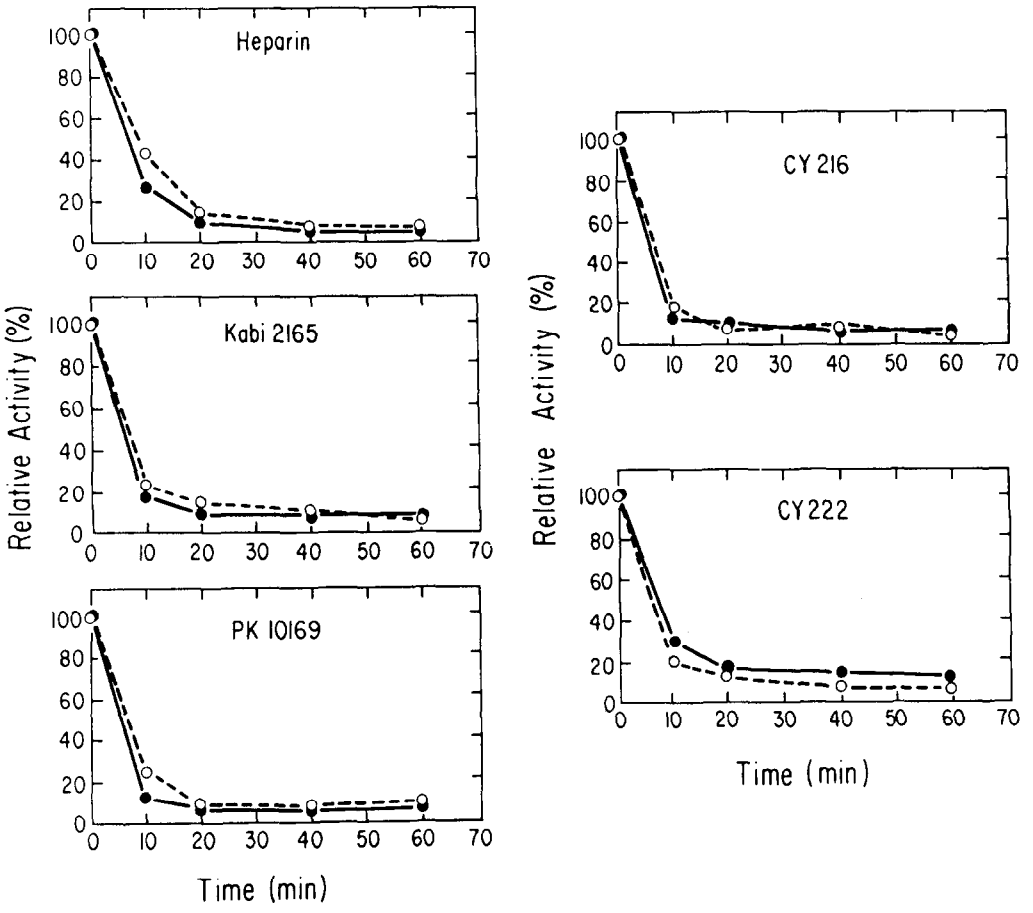

FIG. 2

Removal of the anticoagulant activity of LMHF by immobilized heparinase in human plasma. ●—●—●, APTT activity. O---O---O, anti-Fxa activity. The residual acitvity was represented as the relative activity assuming a 100 % value for the initial activity. For details, see "Methods section.

Figure 1 shows the neutralization of heparin and LMHF by protamine. When protamine was supplemented at 0.03 mg/mL, a complete neutralization of both APTT and Anti-FXa activities was observed for heparin. This value (i.e. the equivalent value) is in good agreement with the figure that is widely accepted and commonly used in clinical practice (i.e. 1 - 2 mg of protamine for each 100 USP units of heparin). The prolongation in APTT and the increase in anti-FXa activity corresponding to the further addition of protamine beyond the equivalent value is a common phenomenon. It is believed to be related to protamine's own anticoagulant properties (20).

Neutralization of LMHF by protamine followed a similar pattern to that of heparin. Both APTT and anti-FXa activities decreased with the increase in protamine concentration until they reached a minimum, at which the protamine concentration is defined as the apparent equivalent value, and then increased with further increases in protamine concentration. However, neither of these activities were ever completely neutralized by protamine for any of the LMHF samples. Even at the apparent equivalent values, at least 40 % of the APTT activity still remained in plasma for each of the cases (Fig. 1). The anti-FXa activity exhibited a stronger resistance to protamine neutralization than that of the APTT activity, and was insensitive to neutralization over a broad range of protamine concentrations. The degree of neutralization, as well as the apparent equivalent value varied with the samples used for testing. These results support the findings reported by other groups that protamine from different sources is not an efficient antagonist to LMHF (10-12).

Removal of Anticoagulant Activity of LMHF by Heparinase in Human Plasma

Figure 2 shows the removal of the anticoagulant activity of heparin and LMHF by heparinase. In all cases including that with normal heparin, more than 80 % of both APTT and anti-FXa activities were abolished in less than 20 min. After 20 min, a nearly complete removal of both activities was noted. The residual activities dropped to a level of less than 7 % of their initial values, and remained unchanged thereafter. Since heparinase was present in a large excess over the level required for complete removal, the kinetics of removal, or in other words, the rate of removal was almost identical for each of the cases despite the different Km values for these LMHF. Since the final degradation products produced by a complete heparinase digestion were reported to possess trace anticoagulant activities (4,10,14), the small amount of APTT and anti-FXa activities (<7 %) remaining in each of the samples after the heparinase digestion of LMHF was believed to be attributed to those of the degradation products.

DISCUSSION

During the past decade, the development of LMHF as new antithrombotic agents or heparin substitutes has gained increased attention. These compounds are characterized with a poor ability to prolong the plasma clotting time and a strong potential to inhibit coagulation Factor Xa (6-9). The potent anti-FXa activity

provides the drugs with their antithrombotic properties, whereas the low APTT activity is believed to significantly reduce the bleeding risks normally associated with conventional heparin therapy (1, 6-9). In addition, the low platelet binding of these agents also enables a reduced tendency of causing thrombocytopenia (21). Moreover, the LMHF exhibit a longer *in vivo* half-life than that of heparin, which may provide them with another benificial feature to permit a low dosage in the prophylactic uses of the antithrombotic agents (21).

Protamine has long been used in conjunction with heparin therapy as a heparin antagonist (22-25). A dosage of 1.3 mg of protamine sulfate generally is used in clinical practice to neutralize 100 USP units of heparin (22). Protamine binds heparin chemically and displaces it from its binding site on the antithrombin molecules, rendering heparin's anticoagulant effects ineffective (22-25). The binding was suggested to require a minimum of 16 saccharide units (11). Since LMHF (MW 2,500 - 5,000 daltons) prepared by depolymerization of normal heparin were composed of 8 - 16 saccharide units, they displayed a very weak affinity for protamine. Several groups have reported the lack of both *in vitro* and *in vivo* efficiency for protamine in neutralizing the anticoagulant effects of LMHF (10-12). Our *in vitro* results are consistent with these findings. When protamine sulfate was supplemented at a dosage most efficient for neutralization (i.e. the apparent equivalent value), more than 40 % of the APTT and 60 % of the anti-FXa activities still remained unneutralized for all the LMHF. These values are slightly higher than those reported by others with the employment of protamine chloride, rather than protamine sulfate, as the neutralizing agent (10,11). The lack of interaction with protamine, as well as the absence of a proper antagonist to LMHF might exclude the LMHF from some major potential therapeutic applications such as in extracorporeal therapy, because of the concern of the bleeding risks associated with these applications where a high dose of anticoagulants are required.

Previously we have suggested an approach which would eliminate bleeding problems associated with extracorporeal heparin therapy (13). This approach consists of placing a blood filter containing immobilized heparinase at the effluent of the extracorporeal device. The filter would convert heparin residing in the extracorporeal device to small saccharides with minimal anticoagulant activity before the blood returned to the patient. Preliminary data showed that such a filter was capable of removing up to 90 % of heparin in a single pass for *in vitro* and *in vivo* uses (13,17). Normal heparin is polydisperse with a molecular weight range from 3,000 to 40,000 daltons (26), and *per se* contains a certain fraction of low molecular weight species. Since heparinase has been shown to degrade this polydisperse heparin indiscriminately to small fragments with an average molecular weight of 1,000 daltons (14), it is interesting to examine whether the above device is capable of neutralizing LMHF. The *in vitro* data confirm this feasibility. LMHF were degraded by heparinase in buffer in a fashion similar to that of heparin, although the kinetics of degradation were somewhat different; this is presumably because of differences in the chemical nature of

these compounds. In plasma, both the APTT and anti-FXa activities of the LMHF were reduced by heparinase to less than 7 % of their intial values within a period of 20 min. The time required for a complete neutralization can be shortened considerably, simply by employing more enzyme in the system. The fact that trace amounts of anticoagulant activities (<7%) still remain in the system after heparinase neutralization is not unanticipated. Heparinase degrades heparin to produce products which have a distribution of approximately 50, 30, 15, and 5 % for di-, tetra-, hexa-, and oligo-saccharide molecules (14). The oligosaccharides have been reported to possess both APTT and anti-FXa activities (4,10,14). The remaining activities are therefore believed to be attributed to those displayed by the oligosaccharides. Although the APTT activity derived from those oligosaccharides was very low, it gave a relatively high value in percentage (13 %) for CY 222 due to the low intial APTT activity (11 units/mg) for this sample.

The immobilized heparinase system presented here appears to be thus far the only means to effectively remove the anticoagulant activities of LMHF. With some modification it also can be used for the in vitro quantitation, and even for the in vitro titration of LMHF. In addition, the heparinase system may assist LMHF in their acceptance for uses in extracorporeal therapy, as well as in their development as improved and "all around" antithrombotic agents. Although the system is believed to be effective in removing LMHF in vivo, animal studies regarding in this area will be conducted.

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