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Effects of S-hexyl-L-cysteine derivatives on prothrombin activation and clotting time determined in the presence of heparin

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ABSTRACT

Purpose: The aim of the study is the examination of effects of dipeptides containing S-hexyl-L-cysteine and glycine, on the prothrombin activation and the thrombin clotting time determined in the presence of heparin.
Material and Methods: The activation of prothrombin was determined with the use of the thromboplastin test, the recalcification and partial thromboplastin with kaolin tests. The thrombin clotting time determined in the presence of heparin was evaluated with the use of the heparin-thrombin test.
Results: The investigated derivatives slightly inhibited the prothrombin activation. The unsubstituted derivatives and dipeptides with a free amino or carboxyl group significantly enhanced the clotting time determined in the presence of heparin at concentration 20 mM.
S-Hexyl-L-cysteinylglycine (H-(S-hexyl)-L-Cys-Gly-OH) was the most active compound.
Conclusions: The obtained results indicate that some dipeptide derivatives of S-hexyl-L-cysteine apart from the earlier observed possibility to prolong the thrombin clotting time, can also prolong the clotting time determined in the presence of heparin.

Key words: S-hexyl-L-cysteine, dipeptide, prothrombin activation, clotting time, anticoagulant, heparin

INTRODUCTION

Pathological clot formation causes many diseases. A number of compounds have been examined as potential anticoagulants [1,2], however the most available and widely studied material is still heparin [3]. During our earlier investigations on biological activity of S-substituted cysteine derivatives, we observed that ε-aminocaproil derivatives of S-methylcysteine and S-benzylcysteine showed antifibrinolytic activity. Similar activity was observed in the case of dipeptides: glycyl-S-benzyl-L-cysteine and S-benzyl--L-cysteinylglycine [4]. We found also that ε–aminocaproil derivatives of S-alkylcysteine with longer aliphatic chain: -butyl, hexyl and nonyl in the high concentration (20mM), instead of expected prolongation of fibrinolysis time in fibrinolytic test, prevented clot formation. ε-Aminocaproil-S-hexyl-L-cysteine was the most active compound [5]. We observed also that glycyl-S-hexyl-L-cysteine prevented clot formation in the range of the examined concentrations [5]. In our previous paper we reported that dipeptide derivatives containing S-hexyl-L-cysteine and glycine prolonged the thrombin clotting time determined with the use of fibrinogen and plasma. They also shortened the time of fibrinolysis or prevented clot formation in the fibrinolytic test [6]. The effect of dipeptides was lower with the use of plasma as a source of clottable fibrinogen than with the use of purified protein itself. S-Hexyl-L-cysteinylglycine was the most active compound in fibrinolytic and anticoagulant tests. The examined dipeptides did not influence the amidolytic activities of thrombin and plasmin [6]. It was impossible to examine S-hexyl-L-cysteine activity because of a problem with solubility under test conditions. The anticoagulant or the fibrinolytic activity of compounds containing S-substituted cysteine was not earlier described. The garlic products (γ-glutamyl--S-alkylcysteines) were an exception [7]. In order to explain

Table 1. **Effect of examined compounds on prothrombin activation.**

Compound	Concentration of compound (mM)	Tromboplastin test (sec)	Recalcification test (sec)	Partial thromboplastin test (sec)
Boc—L—Cys—Gly—OEt S—hexyl	0.2 2.0 20.0	17±1.4 (p=0.2879) 21±1.7** 39±3.1*	135±9.4* 166±11.6* 203±14.2*	52±4.2* 63±5.1* 83±6.6*
Boc—Gly—L—Cys—OMe S—hexyl	0.2 2.0 20.0	21±1.7** 28±2.2* 36±2.9*	133±9.3** 176±12.3* 199±13.9*	46±3.7 (p=1.000) 53±4.2* 75±6.1*
Boc—L—Cys—Gly—OH S—hexyl	0.2 2.0 20.0	17±1.4 (p=0.2879) 20±1.6** 37±2.9*	137±9.6** 180±12.6* 203±14.2*	52±4.2* 60±4.8* 88±7.1*
Boc—Gly—L—Cys—OH S—hexyl	0.2 2.0 20.0	20±1.6** 27±2.16* 32±2.6*	131±9.2 (p=0.2051) 128±8.9* 198±13.9*	44±3.5 (p=0.2051) 56±4.5* 80±6.4*
HCl•H—L—Cys—Gly—OEt S—hexyl	0.2 2.0 20.0	14±1.1 (p=0.0705) 21±1.7** 38±3.1*	134±9* 173±12.* 198±13.9*	51±4.1* 64±5.1* 92±7.4*
HCl•H—Gly—L—Cys—OMe S—hexyl	0.2 2.0 20.0	21±1.7** 29±2.3* 32±2.56*	136±9.5* 120±8.4* 212±16.9*	44±3.5 (p=0.6702) 57±4.6* 78±6.2*
HCl•H—L—Cys—Gly—OH S—hexyl	0.2 2.0 20.0	12±0.9** 22±1.8* 43±3.4*	138±9.7* 216±15.1* 239±16.7*	53±4.2* 68±5.4* 97±7.8*
HCl•H—Gly—L—Cys—OH S—hexyl	0.2 2.0 20.0	21±1.7* 24±1.9* 33±2.6*	136±9.5* 119±8.3* 198±13.9*	44±3.5 (p=0.6702) 60±4.8** 80±6.4**
Control NaCl	0.15 M	17±1.4	130±9.1	46±3.7

** - $p<0.05$; * - $p<0.001$

this unexpected activity and in the search of novel potential anticoagulants, we decided to examine the effect of S-hexyl--L-cysteine derivatives on certain aspects of hemostasis.

The purpose of this work is to determine the influence of eight dipeptides with general formula X-Gly-(S-hexyl)-L-Cys-Y and X-(S-hexyl)-L-Cys-Gly-Y, where X=H, Boc; Y=OH, OMe or OEt, on prothrombin activation and clotting time determined in the presence of heparin.

MATERIALS AND METHODS

Cehalite, BioMerieux France; thrombin and heparin, Sigma USA; thromboplastin, Warszawska Wytwórnia Surowic i Szczepionek, Poland. The examined dipeptides were obtained as described earlier [6]. Human platelet – poor plasma was obtained from citrated blood.

The effects of dipeptides on the prothrombin activation and clotting time determined in presence of heparin were examined as described earlier. Every value represents the average of triplicate determinations ± SD. The results were submitted to the statistical analysis using the Student t-test.

Prothrombin activation

A. The thromboplastin test [8].

0.1 ml of examined compound at concentration 1, 10 and 100 mM (final concentration 0.2, 2 and 20 mM; in control 0.1 ml 0.15 M NaCl) and 0.1 ml of thromboplastin was added to 0.2 ml of plasma. After preincubation for 1 minute at 37°C, 0.1 ml of 0.025 M $CaCl_2$ was added and the clotting time was measured.

B. The recalcification test [8].

0.1 ml of examined compound at concentration 1, 10 and 100 mM (final concentration 0.2, 2 and 20 mM; in control 0.1 ml 0.15 M NaCl) was added to 0.2 ml of plasma. After preincubation for 1 minute at 37°C, 0.2 ml of 0.025 M $CaCl_2$ was added and the clotting time was measured.

C. The partial thromboplastin test with kaolin [9].

0.1 ml of examined compound at concentration 1, 10 and 100 mM (final concentration 0.2, 2 and 20 mM; in control 0.1 ml 0.15 M NaCl) and 0.1 ml of Cephalite were added to 0.2 ml of plasma. After preincubation for 3 minutes at 37°C, 0.1 ml of 0.025 M $CaCl_2$ was added and the clotting time was measured. Results are given in *Tab. 1*.

D. The clotting time in the presence of heparin [10].

0.1 ml of examined compound at concentration 1, 10 and 100 mM (final concentration 0.2, 2 and 20 mM; in control 0.1 ml 0.15 M NaCl) was added to 0.1 ml of heparin solution (0.025

Table 2. **Effect of examined compounds on thrombin clotting time determined in the presence heparin and the clotting time versus control ratio obtained with the use and without heparin.**

Compound	Concentration of compounds (mM)	Clotting time (sec)	Clotting time versus control ratio	
			with heparin	without heparin#
Boc—L—Cys—Gly—OEt S—hexyl	0.2 2.0 20.0	35±2.8 (p=0.7953) 40±3.2* 61±4.9*	1±0.08 1.14±0.09 1.74±0.14	1.36±0.01 1.91±0.14 5.45±0.04
Boc—Gly—L—Cys—OMe S—hexyl	0.2 2.0 20.0	32±2.6** 38±3.1** 59±4.7*	0.91±0.07 1.08±0.08 1.69±0.13	1.18±0.01 1.36±0.14 5.45±0.04
Boc—L—Cys—Gly—OH S—hexyl	0.2 2.0 20.0	33±2.6** 40±3.2* 120±9.6*	0.94±0.07 1.14±0.09 3.43±0.27	1.09±0.01 1.82±0.02 10.18±0.08
Boc—Gly—L—Cys—OH S—hexyl	0.2 2.0 20.0	35±2.8 (p=0.2378) 41±3.3** 115±9.2*	1±0.08 1.17±0.09 3.29±0.26	0.91±0.01 1.09±0.01 24.01±0.17
HCl•H—L—Cys—Gly—OEt S—hexyl	0.2 2.0 20.0	36±2.9 (p=0.7247) 48±3.8* 145±11.6*	1.03±0.08 1.37±0.11 4.14±0.33	0.91±0.01 1.09±0.01 12.54±0.09
HCl•H—Gly—L—Cys—OMe S—hexyl	0.2 2.0 20.0	34±2.7 (p=0.1318) 47±3.8* 128±10.2*	0.97±0.08 1.34±0.11 3.66±0.29	0.82±0.01 1.27±0.01 13.18±0.09
HCl•H—L—Cys—Gly—OH S—hexyl	0.2 2.0 20.0	35±2.8 (p=0.3739) 53±4.2* >200*	1±0.08 1.51±0.12 >5.7	0.91±0.01 1.27±0.01 56.27±0.45
HCl•H—Gly—L—Cys—OH S—hexyl	0.2 2.0 20.0	31±2.5** 41±3.3** >200*	0.89±0.07 1.17±0.09 >5.7	1.18±0.01 1.36±0.02 17.36±0.03
Control NaCl	0.15 M	35±2.8		

#- calculation with the use of earlier data [6]
**- $p<0.05$; * - $p<0.001$

mg/ml). After preincubation for 1 minute at 37°C, 0.2 ml of plasma was added. The incubation was continued for next 3 minutes, 0.1 ml of thrombin (15 U/ml) was added and the clotting time was measured. Results are given in *Tab. 2*.

RESULTS AND DISCUSSION

The investigated derivatives slightly inhibited the prothrombin activation. They prolonged the clotting time about two times at highest examined concentration (20 mM), independently of the method of the prothrombin activation (*Tab. 1*). The inhibitory effect of S-hexyl-L-cysteinylglycine was slightly higher than the other examined compounds, especially in the recalcification test (*Tab. 1*). All examined compounds enhanced the thrombin clotting time determined in the presence of heparin (*Tab. 2*) but the effect of fully substituted dipeptides was lower than determined without heparin. A higher activity was observed in the case of partially protected compounds and there was no difference which one from the groups: amino or carboxyl was substituted. Dipeptides with free amino and carboxyl groups showed higher enhancement of the clotting time, observed at the highest examined concentration (20 mM). S-Hexyl-L-cysteinylglycine was the most active compound but the activity of glycyl-S-hexyl-L-cysteine was also significant (*Tab. 2*). According to the results obtained earlier, this group of compounds does not rather affect the active centres of thrombin and plasmin, because they do not influence the synthetic substrate hydrolysis [6]. The examined dipeptides also cannot interact with thrombin exosites because they require peptide derivatives with acidic residues [11]. Their anticlotting and fibrinolytic activities [6] can probably be connected with an interaction with fibrinogen or fibrin monomers. The shortening of the clotting time or the prevention of the clot formation are connected with the presence of S-alkyl derivative of cysteine with a long aliphatic chain in dipeptide strukture. S-Hexyl group was the most effective [5]. We have observed that S-hexyl-L-cysteine containing dipeptides enhanced the clotting time determined in the presence of heparin (*Tab. 2*). In this case the polarity of the examined compounds is very important. Only dipeptides with free amino and carboxyl groups showed the considerable activity in presence of heparin. In experiments

without heparin also partially protected dipeptides showed significant enhancement of thrombin clotting time in highest concentration 20 mM. The problem whether S-hexyl derivatives of L-cysteine can be potential anticoagulant drugs needs further research.

CONCLUSIONS

The obtained results indicate that some dipeptide derivatives of S-hexyl-L-cysteine apart from the earlier observed possibility to prolong the thrombin clotting time, can also enhance the clotting time determined in the presence of heparin but only compounds with free amino and carboxyl groups show the considerable activity.

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