Novel therapeutic agents designed as a complementary peptide to target molecules

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Abstract: We generated an evolutionary computer program that generates complementary peptide (C-pep) sequences with potential to interact with a target peptide by comparing several physic-chemical parameters of each pair of the complementary peptides being analyzed. We generated C-peps to several target molecules. About 30% of synthesized C-peps interfered with the function of their targets. C5a stimulates generation of TNF□ and other inflammatory cytokines. Inhibition of C5a should be effective on sepsis which impair the status of cancer bearing patients. One of the inhibitory C-pep of C5a termed AcPepA was effective in cynomolgus monkeys intravenously infused with a lethal dose of bacterial LPS (4mg/kg) destined to death. The monkeys were rescued by intravenous administration of 2 mg/kg/h of AcPepA. The excellent therapeutic effect of AcPepAshould be due to restriction of high mobility group box 1 (HMGB1) surge to be induced by effect of C5a on C5L2 which is the second C5a receptor.

After proposal of the possible role of antisense peptides for molecular interaction among proteins by Blalock et al. (1) in 1984, the theory was reviewed later (2,3). Many examples of sense-peptide and antisense-peptide relationships have been found between receptors and their protein ligands (4-8). We speculated that such interactions between sense- and antisense- peptides should play a role in formation of the tertiary structure of proteins. We developed a novel computer program named ANTIS to find antisense peptide sequences between proteins to be compared (9). By analyzing intra-molecular antisense peptides within a single protein molecule, we found that there are an appreciable number of sense- and antisense-peptide pairs within a protein molecule and we

designated these as antisense homology boxes (AHB) (9). Using ANTIS, we analyzed sense- and antisense-peptide relationships in the endothelin receptor (ETR) molecule and between endothelin and ETR. One of the AHB peptides of ETR named ETR-P1/fl had a capacity to interfere with the function of ETR (10). Then we expected that it will be possible to generate candidates of complementary peptide reactive with target amino acid sequence basing upon sense-antisense amino acid relationship. We generated an evolutionary computer program that runs on any PC computer, and generates complementary peptide sequences with potential to interact with a target peptide by comparing several physic-chemical parameters of each pair of the complementary peptides being analyzed (11). With the program named MIMETIC, we generated complementary peptides to HIV-reverse transcriptase (11,12), procarboxypeptidase R, thrombomodulin (13), and C5a anaphylatoxin (14) as listed in Table 1. About 30% of synthesized peptides interfered with the function of their target molecules. Among them, the complementary peptides (C-pep), out of 19 C-pep targeted to C5a anaphylatoxin, 7 showed inhibitory effect.

C5a is a 74-amino acid peptide released from the fifth component of complement (C5) by C5 convertase generated during complement activation (15). C5a anaphylatoxin is considered to be an effective target for treatment of hyper inflammation since C5a stimulates generation of TNFα and other inflammatory cytokines (16-18). Although C5a generated in vivo is regulated by carboxypeptidase N and more efficiently by carboxypeptidase R (CPR) (19,20), excessive generation of C5a appears to exceed the capacity of CPR, since administration of LPS at a lethal dose to rats exhausted CPR capacity before death (21). However, attempts to restrict the effect of C5a with C5a receptor (C5aR) antagonists would not be successful because C5aR is expressed not only on inflammatory leukocytes, but also on many other cell types (17). Furthermore C5aR numbers increase in an acute inflammatory state (22).

On the other hand, antibodies to C5a have been demonstrated to be effective in treating experimental septic primate models (16,23) indicating that C5a inhibitors should be useful for treatment of patients suffering from hyper-inflammation as in sepsis and multiple organ failure (24). If an inhibitor of C5a has a therapeutic effect on sepsis which impair the status of cancer bearing patients, the inhibitor could be beneficial for the cancer patients to improve performance status.

Antisense Homology Box (AHB) in C5a receptor (C5aR), and between C5aR and C5a was analyzed by ANTIS program, and we found that amino acids 37 to 53 of C5a (RAARISLGPRCIKAFTE) is an antisense peptide to AHB peptides (9) of the C5aR, and has been designated PL37 (25). This region of C5a is presumed to be a potential site for C5aR stimulation (26). Using the computer program, MIMETIC (11), we generated 19 complementary peptide (C-pep) to PL37. One of the 7 inhibitory C-pep to PL37 which interfered with C5a function was

termed PepA (ASGAPAPGPAGPLRPMF) (14). To improve stability, we modified PepA by acetylation of its N-terminal alanine generating acetylated PepA (AcPepA) which was more stable in animal experiments (27). In preliminary experiments with human lung tissues, AcPepA successfully suppressed the allergic response in vitro (28). Therefore, we performed experiments in cynomolgus monkeys in lieu of using human subjects.

Materials and Methods

Peptides. PepA (ASGAPAPGPAGPLRPMF) whose N-terminal alanine is acetylated (AcPepA) was synthesized and purified (over 95% purity) by Biologica Co. Ltd. (Nagoya, Japan). The peptide was dissolved in saline at a concentration of 2mg/ml and passed through a 0.22 μm Millipore filter prior to administration intravenously with an automated injection pump.

Momkeys. Cynomolgus monkeys were supplied from a breeding colony maintained at the Corporation for Production and Research of Laboratory Primates (CPRLP), Tsukuba, Japan. The Institutional Animal Ethical Committee of the Choju Medical Institute, Fukushimura Hospital, and the Institutional Animal Care Use Committees of the Tsukuba Primate Research Center, National Institute of Biomedical Innovation approved the study protocol. Animals weighed 4 to 5.5 kg, had hematocrits exceeding 36% and were free of infection including tuberculosis. Animals were held for one month prior to LPS-lethal shock studies at CPRLP.

Titration of cytokine levels in plasma. TNFα in monkey plasma was determined using an ELISA kit purchased from Quantikine Immunoassay (Minneapolis, MN). MIF was determined by use of an ELISA kit (29) prepared by Sapporo Immuno Diagnostic Laboratory (Sapporo, Japan). For HMGB1 determination, an ELISA kit from Shino-Test Co. (Sagamihara-shi, Kanagawa, Japan) was used.

Results

Monkeys were administered a lethal dose of LPS (4mg/kg) sufficient to kill a monkey within 2 days. Following sedation using ketamine hydrochloride (14mg/kg, subcutaneously), monkeys were anesthetized with sodium pentobarbital administered through the capalic vein via a percutaneous catheter to maintain light level surgical anesthesia. Oral intubation allowed animals to breathe spontaneously. Under anesthesia with sodium pentobarbital, monkeys were intravenously administered 4 mg/kg LPS within 30 min. Six hours after the LPS administration, anesthesia

treatment was terminated and monkeys were returned to their cages to observe their status without any additional interference.

Although all 3 monkeys administered saline alone as an untreated control died within 2 days (two in one day and one in two days), administration of 2mg/kg of AcPepA in 2 min followed by 2 mg/kg/hr of AcPepA for 3 hrs starting 30 min after the LPS injection rescued all of 7 monkeys who returned to a healthy condition in 2 days (Table 2). Following LPS administration, significant leukopenia and thrombopenia were observed in monkeys treated with AcPepA as well as untreated saline control monkeys in peripheral blood obtained 6 hrs after the LPS injection (Figure 1). However, the TNFα level in plasma obtained during experiments in treated monkeys decreased by only about 30% compared with that of untreated monkeys (Figure 2). The increase in the level of macrophage migration inhibitory factor (MIF) (Figure 3) and high mobility group box 1 (HMGB1) (Figure 4) after LPS injection tended to be suppressed in the AcPepA-treated monkeys. Some of the monkeys were sacrificed under anesthesia 6 hrs after LPS in order to perform autopsies. Pathological analysis of organ tissues showed serious inflammatory changes including leukocyte infiltration to the same extent in the lungs of both treated and untreated monkeys (Figure 5).

Discussion

The AcPepA treated monkeys might have escaped induction of a feedback inflammatory circuit progressing gradually in LPS treated monkeys at a late stage of the endotoxin shock syndrome (Figure 6). This may be because HMGB1 has the capacity to stimulate TLR4 and TLR2 as an endogenous stimulator (30,31). Consequently, inhibition of HMGB1 release has presumably rescued animals suffering from septic syndrome (32,33). Therefore, suppressed HMGB1 in monkeys treated with AcPepA (Figure 4) could explain the therapeutic effect of AcPepA on endotoxin shock animals (34). In other words, continuous generation of C5a by LPS or bacteria in LPS-shock monkeys as well as possibly in septic patients likely induce a cytokine storm amplified by released HMGB1 resulting in lethal effect on the host. The suppression of HMGB1 induction by inactivation of C5a could directly correlate with the survival observed following AcPepA treatment of monkeys injected with a lethal dose of LPS. Furthermore, AcPepA could suppress pathophysiological events and prolonged survival time of sepsis piglets induced by cecal ligation and perforation (CLP) (35,36). Survival times were longer in the AcPepA treated group than in the CLP alone group (19.3hrs ± 2.7hrs vs. 9.9 hrs ± 0.7 hrs, $P<0.005$). In this case, AcPepA also delayed the

HMGB-1 surge (36).

Therefore, suppression of C5 anaphylatoxin by AcPepA interferes with the induction of a cytokine storm. Since C5a has the capacity to cause release of HMGB1 following stimulation of the second C5a receptor termed C5L2 generated on activated monocytes (37-39), inhibition of C5a successfully interferes with the above release which has the capacity to generate inflammatory cytokines stimulating TLR4 as an endogenous ligand (Figure 7) (34).

Therefore, AcPepA would be beneficial for treatment of septic patients and could be administered in large amounts at an acute stage with little likelihood of an overdose since the half-life of AcPepA in rats is 2.5 min. Administration of AcPepA to cancer patients at their terminal stage could improve their performance status suppressing cytokine storm.

Acknowledgments

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Table 1. Inhibitory capacity of complementary peptides (C-pep) to target molecules. Complementary peptides designed by MIMETIC program were synthesized and determined their inhibitory capacity on the function of target melecules. About 30% of them interfered with the activity of target molecules.

Target molecule	Activity of target	Number of C-pep	
		Tested	Effective (%)
HIV-RT*	Enzyme activity	10	3 (30%)
ProCPR **(TAFI)	Enzyme activity	10	3 (30%)
Thrombomodulin	Cofactor activity***	3	2 (67%)
C5a anaphylatoxin	Bioactivity	19	7 (37%)

*RT: reversetranscriptase (12)

**ProCPR: procarboxypeptidase R

***Cofactor activity for thrombin (13)

Table 2. Therapeutic effect of AcPepA on monkeys inoculated with a lethal dose of LPS (4mg/kg)

	LPS alone	LPS and AcPepA	AcPepA alone
Decreased BP	4/6	3/7	0/1
Increase in body temperature	5/6	3/7	0/1
Leukopenia	6/6	7/7	0/1
Increase CPK	6/6	7/7	0/1
Death	3/3	0/7	0/1
	(3/3)*		

* Three monkeys were euthanized to prevent pain prior to death.

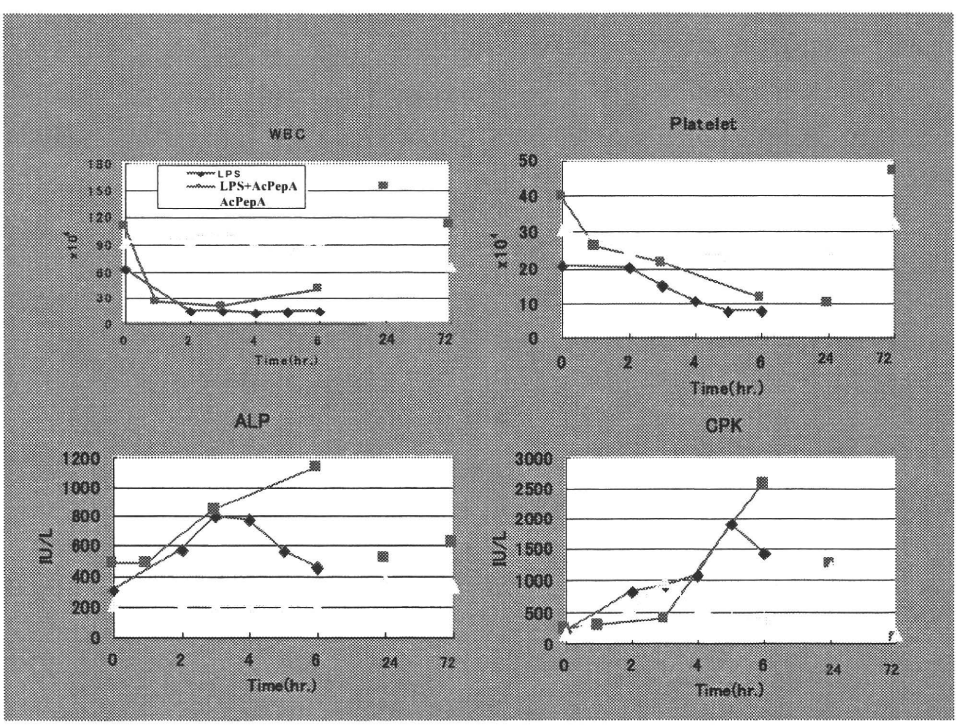

Figure 1. Clinical features in peripheral blood of endotixin-shock monkeys. Extensive leukopenia (WBC), and thrombopenia (Platelet), increased alkaline phosphatase (ALP) as well as increased creatinin phosphokinase (CPK) were observed in blood from AcPepA treated monleyes following LPS injection as in the case of untreated monkey. Esssentially no significant changes were observed in a monkey treated with AcPepA alone without LPS injection.

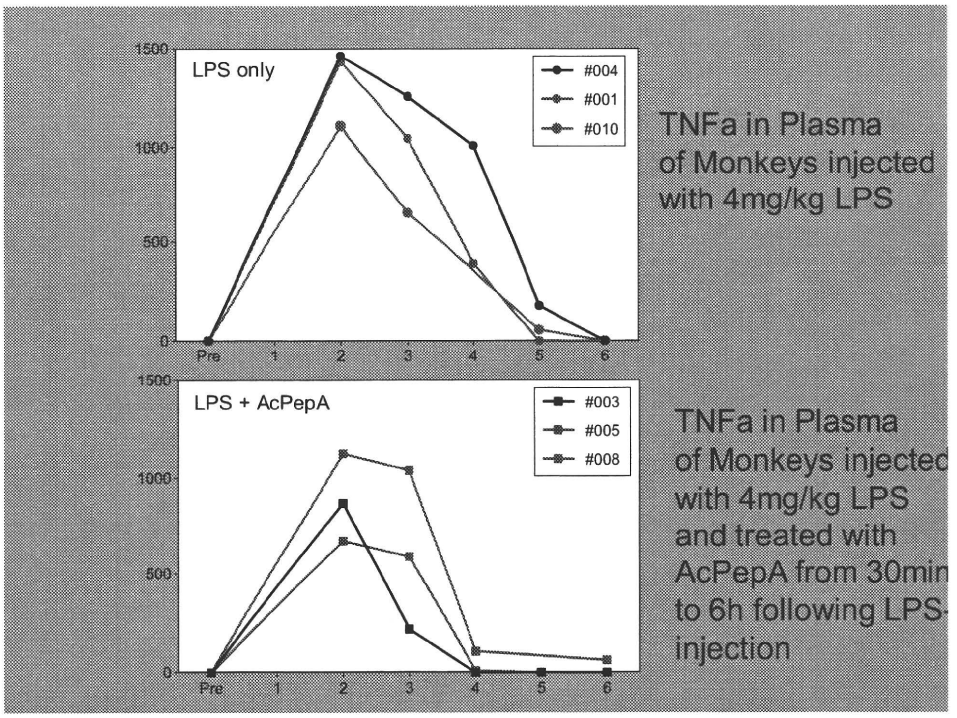

Figure 2. TNFα levels in plasma of monkeys injected with 4mg/kg LPS (upper panel) and monkeys treated with AcPepA following the LPS injection (lower panel).

AcPepA treatment suppressed the TNFα level to approximately 60% of the untreated ones. TNFα in plasma disappeared within 4 hrs after LPS injection in AcPepA treated monkeys whereas it took 5 hrs in untreated monkeys.

試 験 計 画 書

試験表題：　LC/MS/MS によるサル血漿中 AcPepA 中濃度測定法バリデーション

試験番号：　PBC861-001

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略号及び用語の一覧

略号／用語	定義
C.V.	Coefficient of variation（変動係数，精度）
EDTA	Ethylenediaminetetraacetic acid
ESI	Electro spray ionization
GLP	Good Laboratory Practice
HPLC	High performance liquid chromatography
LC/MS/MS	Liquid chromatography / tandem mass spectrometry
LLOQ	Lower limit of quantification（定量下限）
MRM	Multiple reaction monitoring
MS/MS	Tandem mass spectrometry
PFA	*p*-Fluorophenylalanine
PP	Polypropylene
QAU	Quality Assurance Unit（信頼性保証部門）
r	Correlation coefficient（相関係数）
R.E.	Relative error（相対誤差，真度）
S.D.	Standard deviation（標準偏差）
SNBL DSR	Shin Nippon Biomedical Laboratories, Ltd., Drug Safety Research Laboratories（株式会社新日本科学　安全性研究所）
SNBL PBC	Shin Nippon Biomedical Laboratories, Ltd., Pharmacokinetics and Bioanalysis Center（株式会社新日本科学　薬物代謝分析センター）
SOP	Standard Operation Procedure（標準操作手順書）
TFA	Trifluoroacetic acid（トリフルオロ酢酸）
ULOQ	Upper limit of quantification（定量上限）

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1　試験表題

LC/MS/MS によるサル血漿中 AcPepA 中濃度測定法バリデーション

2　試験目的

LC/MS/MS によるサル血漿中 AcPepA の濃度測定法の信頼性を確認することを目的として実施する．また，血漿中 AcPepA の安定性を確認する．

3　適用規制

本試験は GLP 非適用であるが，「申請資料の信頼性の基準」（薬事法施行規則第 43 条：平成 17 年 3 月 23 日厚生労働省令第 37 号）を遵守して実施する．

また，試験計画書，生データ及び最終報告書の整合性について，QAU による信頼性調査を行う．

4　試験委託者

医療法人さわらび会福祉村病院　長寿医学研究所

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5　試験施設

SNBL PBC

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6　試験関係者

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分析担当者：　　阿部　直樹，建川　一朗

被験物質管理責任者：　　近藤　貴雄

7　日程

試験開始日：　　2011 年 1 月 20 日

測定開始予定日：　　2011 年 1 月 20 日

測定終了予定日：　　2011 年 5 月 13 日

最終報告書草案作成日：　　2011 年 6 月 24 日

試験終了日（最終報告書作成日）：2011 年 7 月 28 日

8　材料及び方法

8.1　使用機器及び材料

以下の装置，機器又はその同等品を使用する．

装置又は機器	型式	製造者又は販売業者	SOP
HPLC システム	島津 10A システム	株式会社島津製作所	CALC/232
	島津 20A システム		
MS/MS システム	API 3000	AB SCIEX	CALC/202
データ処理ソフト	Analyst		
天秤	AT261	メトラー・トレド株式会社	CALC/302
	AX205		
	AE240		
	CP225D	ザルトリウス・メカトロニクス・ジャパン株式会社	
冷却遠心機	LX-120	株式会社トミー精工	CALC/308
	MX-301		
超純水製造装置	Milli-Q SP	日本ミリポア株式会社	CALC/314
	Milli-Q Gradient-A10		
窒素乾固装置	Turbo Vap LV Evaporator	Caliper Life Sciences, Inc.	CALC/322
可変容量型ディスペンサー	Eppendorf	Eppendorf AG	CALC/324
	Finnpipette	Thermo Fisher Scientific K.K.	
	Finnpipette P.C.R. (PDP)		RILC/345
	Microman pipette	Gilson, Inc.	RILC/369
冷凍室	LVF4JA	ダイキン工業株式会社	CALC/006
冷蔵室	LVL1X5KA	ダイキン工業株式会社	CALC/006
冷凍冷蔵庫	MPR-414FRS	三洋電機株式会社	TSBC/004
冷凍庫	MDF-436	三洋電機株式会社	TSBC/007
固相抽出カートリッジ	Oasis HLB (1 mL, 30 mg)	Waters	-

8.2　標準物質，内標準物質，ブランク試料及び試薬

（SOP：CALC/003，CALC/005，TSBC/001，TSBC/007，RILC/108）

標準物質及び内標準物質は被験物質管理責任者が受領・保管する．

試験で必要な量を分取し，本試験内で用いる．分取しなかった分は引き続き被験物質管理責任者が保管する．

8.2.1　標準物質

名称：	MPS-390（AcPepA）
送付元：	医療法人さわらび会福祉村病院　長寿医学研究所
ロット番号：	2K09030
受領日：	2010 年 11 月 17 日
受領量：	200 mg
含量：	97.7%
使用期限：	委託者より安定性に関する情報を入手する
保存条件：	冷凍，遮光，気密
保存場所：	被験物質保管室 2　冷凍庫（冷凍，許容範囲：−30〜−10°C）
取扱い：	手袋，キャップ，保護メガネ及びマスクを着用する．

8.2.2　内標準物質

名称：	Labeled [$^{15}N_6$]-AcPepA
送付元：	オペロン　バイオテクノロジー株式会社
ロット番号：	1011089M（ID）
受領日：	2011 年 1 月 17 日
受領量：	50 mg（10 mg×5 本）
純度：	97.63%
安定性	2012 年 1 月
保存条件：	冷凍，遮光，気密
保存場所：	被験物質保管室 2　冷凍冷蔵庫（冷凍，許容範囲：−40〜−20°C）
取扱い：	手袋，キャップ，保護メガネ及びマスクを着用する．

8.2.3　残余標準物質及び内標準物質の処理

被験物質管理責任者に返却する．

8.2.4　ブランク血液及び血漿

動物種：	カニクイザル（雌雄）
抗凝固剤：	EDTA・2K
供給元：	SNBL DSR

SNBL PBC

保存条件：　血液：冷蔵（許容範囲：1～8°C）
血漿：冷凍（許容範囲：−30～−10°C）

8.2.5　試薬

以下の試薬又はその同等品を使用する.

試薬	等級	製造者
アセトニトリル	HPLC 用	和光純薬工業株式会社
ぎ酸	試薬特級	和光純薬工業株式会社
TFA	試薬特級	和光純薬工業株式会社
超純水	蒸留水を超純水製造装置で精製する.	

8.3　標準溶液の調製

検量線用標準原液及び標準溶液とバリデーション用標準原液及び標準溶液はそれぞれ別途に調製する.

8.3.1　標準原液

下記のとおり標準物質を 30 vol%アセトニトリル溶液に溶かして検量線用標準原液及びバリデーション用標準原液を調製する.

標準物質名：　AcPepA
秤取量：　12.5 mg
調製量：　25 mL
調製濃度：　500 µg/mL
使用溶媒：　30 vol%アセトニトリル溶液
使用器具：　PFA 製メスフラスコ
保存条件：　冷蔵（許容範囲：1～8°C）
保存場所：　冷蔵室
使用期限：　本試験内で確認する.

8.3.2　標準溶液

次表のとおり，検量線用標準原液及びバリデーション用標準原液より順次希釈し，検量線用及びバリデーション用標準溶液を調製する．PP 製容器を使用する.

検量線用標準溶液

標準溶液 No.	使用標準溶液		30 vol%アセトニトリル溶液量 (mL)	調製濃度 (ng/mL)
	No.	採取量（mL）		
SS8	検量線用標準原液	1	9	50000
SS7	SS8	1	9	5000
SS6	SS7	4	6	2000
SS5	SS7	2	8	1000
SS4	SS6	2	6	500
SS3	SS5	2	8	200
SS2	SS4	2	8	100
SS1	SS3	2	6	50

使用溶媒：　30 vol%アセトニトリル溶液
保存条件：　冷蔵（許容範囲：1～8°C）
保存場所：　冷蔵室
使用期限：　本試験内で確認する.

バリデーション用標準溶液

標準溶液 No.	使用標準溶液		30 vol%アセトニトリル溶液量 (mL)	調製濃度 (ng/mL)
	No.	採取量（mL）		
SV6	バリデーション用標準原液	1	9	50000
SV5	SV6	1	9	5000
SV4	SV6	1	11.5	4000
SV3	SV4	1	7	500
SV2	SV3	2	8	100
SV1	SV3	1	9	50

使用溶媒：　30 vol%アセトニトリル溶液
保存条件：　冷蔵（許容範囲：1～8°C）
保存場所：　冷蔵室
使用期限：　本試験内で確認する.

8.4　内標準溶液の調製

8.4.1　内標準原液

下記のとおり内標準物質を 30 vol%アセトニトリル溶液に溶かして内標準原液を調製する.