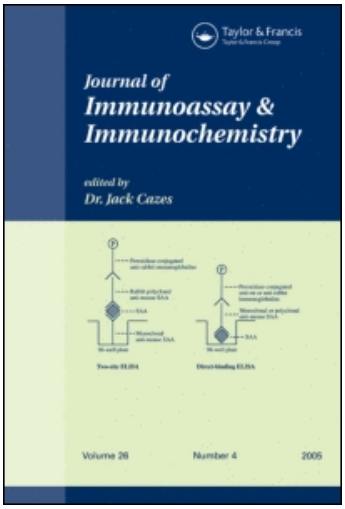

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DEVELOPMENT OF AN ENZYME IMMUNOASSAY FOR A STABLE AMIDATED ANALOG OF THE HEMOREGULATORY PEPTIDE ACETYL-SER-ASP-LYS-PRO

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ABSTRACT

The tetrapeptide Acetyl-Ser-Asp-Lys-Pro (AcSDKP) has been shown to protect hematopoietic stem cells from the toxicity of anticancer chemotherapies. Since its pharmacological efficacy is limited by a rapid degradation by Angiotensin-I Converting Enzyme (ACE), AcSDKP analogs resistant to ACE have been synthesized. One of these compounds (AcSDKP-NH,) differs from the native AcSDKP by amidation of the C-terminus. Further evaluations of this molecule require an analytical method in order to characterize its pharmacokinetic profile. We report, here, the development of a highly specific and sensitive enzyme immunoassay (EIA) for AcSDKP-NH, that

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does not cross-react with endogenous or exogenous AcSDKP. Using AcSDKP-NH_2-acetylcholinesterase conjugate as a tracer, rabbit specific antiserum and microtiter plates coated with goat anti-rabbit immunoglobulins, this EIA allows the determination of AcSDKP-NH_2 with limits of quantitation of 1 nM in mouse plasma and 100 pmol/g in tissues.

Intra-day and inter-day coefficients of variations were less than 20%. The method was successfully applied to a pharmacokinetic study in order to compare plasma and tissue profiles of AcSDKP-NH_2 and AcSDKP. Plasma AcSDKP-NH_2 levels were found higher than those of AcSDKP, with AUC_{inf} and C_{max} values, respectively, 26- and 10-fold higher than that of AcSDKP.

INTRODUCTION

Isolated from fetal calf bone marrow, the tetrapeptide Acetyl-Ser-Asp-Lys-Pro (AcSDKP) has been identified as a negative regulator of hematopoiesis.(1) It inhibits *in vivo,* as well as *in vitro*, the proliferation of human and murine primitive hematopoietic cells.(1-4) *In vivo,* AcSDKP protects hernatopoietic stem cells and early progenitors against the toxicity of anticancer drugs (5, 6) or irradiation (7, 8) and may be used as a myeloprotective agent during anticancer therapeutic regimens.(9)

However, AcSDKP is rapidly hydrolyzed *in vitro*(10) and *in vivo*(11) by Angiotensin I-Converting Enzyme (ACE), and its short half-life(12) may compromise its clinical use. To overcome this drawback, stable AcSDKP analogs resistant to ACE degradation have been designed(13) and successfully evaluated *in vitro*.(14) One of these analogs, hereafter called AcSDKP-NH_2, only differs from the native AcSDKP in its amidated C-terminus proline (Figure 1).

Further evaluations of the therapeutic efficacy of AcSDKP-NH_2 require the development of an analytical method for the characterization of its pharmacokinetic profile, and for the establishment of pharmacokinetic-pharmacodynamic relationships so as to correlate the biological activity of this drug with its plasma or tissue concentrations. Because of their sensitivity, simplicity, and high throughput, immunoassays (EIA) are a reference methodology for the measurement of peptides, therapeutic proteins, or biomarkers in biological media.(15) AcSDKP, which has been reported to be ubiquitously distributed in mice and at high concentrations,(16) could interfere with the AcSDKP-NH_2, determination by EIA. We, therefore, report here the development of a highly specific competitive EIA that can discriminate between AcSDKP-NH_2, (C-terminus amidated

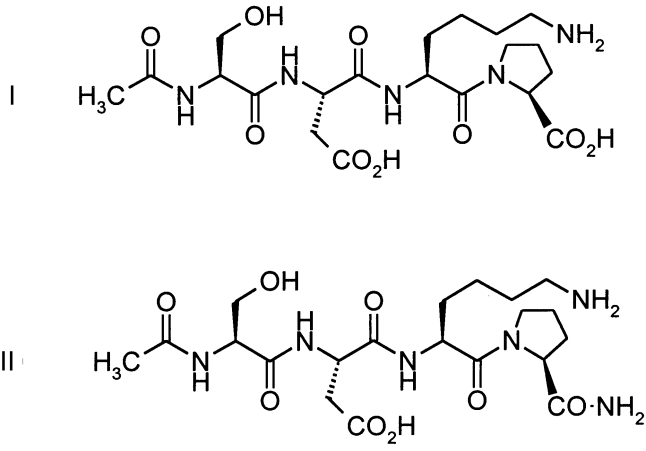

Figure 1. Structure of AcSDKP (I) and AcSDKP-NH_2 (II).

AcSDKP) and endogenous AcSDKP, and its application to a pharmacokinetic study in order to compare plasma and tissue concentrations of these two molecules.

EXPERIMENTAL

Chemicals

Acetyl-Ser-Asp-Lys-Pro was from Neosystem (Strasbourg, France). The C-tenninus modified peptide Acetyl-Ser-Asp-Lys-Pro-NH_2 was synthesized as previously described.(13) All the other peptides used in this study were synthesized in our laboratory, by using the mixed anhydride methodology.(17) The purity, assessed by reverse phase HPLC (C_{18} Waters Nova-Pack column, 4 mm, 3.9 x 150 mm) and by electrospray mass spectrometry analysis (Fisons VG ZabSpec-T), was above 90%.

HPLC grade acetonitrile and methanol were from Merck (Darmstadt, Germany). Bovine serum albumin, lisinopril, 25% glutaraldehyde, sodium azide, and trifluoroacetic acid were from Sigma (Saint Louis, MO). Acety1cholinesterase (AChE) (E.C.3.1.1.7) was extracted from the electric organ of *Electrophorus electricus,* and was purified by one-step affinity chromatography as described elsewhere.(18) The use'of the G4 form of the enzyme for the synthesis of enzymatic tracers used in enzyme immunoassays has been patented by our laboratory. The purified enzyme is available from

SPI-BIO (Massy, France). Enzyme activities were measured using Ellman's reagent, an AChE substrate comprising 2.2 g of acetylthiocholine and 1 g of dithiodinitrobenzene in 200 mL of 0.05 M phosphate buffer pH 7.4. One Ellman unit is defined by the concentration of enzyme producing an absorbance increase of 1 during 1 min in 1 mL of substrate medium for an optical path length of 1 cm.

Immunogen Preparation and Immunization (Antiserum Preparation)

Five μL of 25% glutaraldehyde were added to 2 μmoles of AcSDKP-NH_2 and 12 mg of BSA in 1.0 mL of 0.1 M phosphate buffer pH 7.4. The reaction mixture was incubated for 120 minutes at room temperature and centrifuged. The supernatant was used for immunization. To evaluate the coupling efficiency, the product of the coupling reaction was analyzed by MALDI-TOF mass spectrometry in a Voyager DE RP instrument (PE Biosystems, Les Ulis, France). Sinapinic acid was used as matrix.

Three white rabbits (Blanc du Bouscat, Evic, France) were immunized and boosted with the immunogen (70-80 μg of coupled peptide) by intraderinal injections, using complete Freund's adjuvant. The rabbits were bled 15 days and 30 days, after the boosters given every month, with 35-40 μg of coupled peptide. The bleedings were kept at 4°C after the addition of sodium azide (0.01% final).

Enzymatic Tracer Preparation

Tracer was obtained by conjugation of AcSDKP-NH_2 to acety1cholinesterase (AChE). Thiol groups were first introduced into AcSDKP-NH_2 by reaction of AcSDKP-NH_2 with succinimidyl-S-acetyl-thioacetate (SATA) (20 μmol) in 1 mL of dimethylfonnamide. Activated AcSDKP-NH_2 was purified on a Sep-Pak column previously washed successively with 5 mL of methanol and 10 mL of 0.1% TFA. After application of 600 μL of the reaction mixture, the column was washed with 30 mL of 0.1% TFA and the desired activated AcSDKP-NH_2 was eluted with 5 mL of acetonitrile. After evaporation to dryness, activated AcSDKP-NH_2 (500 nmole) was resuspended in 0.450 mL of 0.1 M borate buffer pH 6.0. Thioester groups were hydrolyzed by addition of 0.05 mL of 1 M hydroxylamine (pH 7.4). Thiol-containing AcSDKP-NH_2 (10 nmol) was mixed with AChE (0.1 nmol) previously activated with N-succinimidyl-4 (N-maleimidomethyl) cyclohexane 1-carboxylate and incubated over night at $+4$°C. Enzymatic tracer was purified by means of molecular sieves chro-

matography using a Biogel A 1.5 (Biorad, Paris, France) column (90 x 1.5 cm) eluted with 0.1 M phosphate buffer pH 7.4 containing 0. 15 M NaCl, 5 mM EDTA, 0. 1 % BSA and 0.01% sodium azide. The collected fraction (2 mL) exhibiting AChE activity (60 Ellman units) was stored at +4°C.

Enzyme Immunoassays

For AcSDKP-NH_2 determination, 96 well microtiter plates (Nunc, Denmark) were coated with mouse monoclonal antibodies specific for rabbit IgG (Spi-Bio, Massy, France). Before use, coated plates were washed with 0.01 M phosphate buffer (pH 7.4) containing 0.05% Tween 20 (washing buffer) using the Autowash 96 (Labsystems, 300 μL/well and five wash cycles). The dilution buffer for tracer and antiserum was 0. 1 M phosphate buffer pH 7.4 with 0.15 M NaCl, 5 mM EDTA, 0.1% BSA, and 0.01% sodium azide (EIA buffer). Standard and quality control samples were diluted in drug-free human plasma (Etablissement de Transfusion Sanguine (ETS), Rungis, France).

The assay was performed in a total volume of 150 μL. Reagents were dispensed as follows: 50 μL of sample, quality control, or standard, 50 μL of tracer and 50 μL of antiserum. After incubation at 4°C for 24 h, the plates were washed as described above and Ellman's reagent (200 μL) was dispensed into each well and incubated in the dark without agitation. When the absorbance in the wells corresponding to the enzyme activity in absence of competitor reached 0.2-0.4, the absorbance at 414 nm was measured in each well using a Multiskan Spectrophotometer (Labsystems, Les Ulis, France).

Unknown concentrations were calculated from a standard curve modeled with a cubic spline transformation (Immunofit, Beckman, Gagny, France). All measurements for standards and samples were made in duplicate. Non-specific binding was determined in wells in which the antiserum was replaced by 50 μL of EIA buffer.

AcSDKP was determined in plasma and tissue samples by a competitive enzyme immunoassay(19) which has been previously used for human(12) and animal(20) pharmacokinetic studies. The antiserum to AcSDKP did not cross-react with AcSDKP-NH_2 (cross-reactivity less than 0.06%).

Validation of the Enzyme Immunoassay for AcSDKP-NH_2

Validation of the enzyme immunoassay for AcSDKP-NH_2 included the range of reliable response, specificity, limit of quantitation, precision,

accuracy, and sample stability. The range of reliable response was established using an eight-point standard curve in human plasma. The specificity of the immunoassay was assessed by a cross-reactivity study with AcSDKP, structural analogs, and potential degradation products. Cross-reactivity coefficients were determined by comparing the molar concentration at 50% B/B_0 of each peptide to that of AcSDKP-NH_2 and expressing it as a percentage of AcSDKP-NH_2 immunoreactivity.

The limit of quantitation, established by analyzing 8 individual mouse plasmas spiked with AcSDKP-NH_2, corresponded to the lowest concentration of AcSDKP-NH_2 allowing good assay precision (CV of repeatability and reproducibility less than 20%) and acceptable accuracy (accuracy ratio in the range of 85 to 115%). The stability was evaluated by analyzing AcSDKP-NH_2 in plasma samples stored at 4 and 20°C for 24 hours, at 37°C for 3 hours, and after 3 freeze-thaw cycles. The stability of AcSDKP-NH_2 in tissue extracts was evaluated under the same experimental conditions at 4 and 20°C. All measurements were performed in triplicate.

Assay precision was estimated in terms of repeatability (intra-assay precision) and reproducibility (inter-assay precision). Repeatability was estimated in terms of the coefficient of variation (CV) for 3 quality control samples assayed five times in the same run. Reproducibility was estimated in terms of the CV for quality control samples assayed in five independent runs. Accuracy was calculated as the ratio between the measured and theoretical concentrations, multiplied by 100.

Pharmacokinetic Studies

CBA/J mice (6-8 weeks old) from Iffa-Credo (Saint Aubin-les-Elbeufs, France) were maintained on a 12-h light-dark cycle, with light from 7:00 a.m. to 7:00 p.m., in a temperature (21 to 22°C and humidity (50%±10%) controlled room. The mice were treated after a 1 week acclimation period. The studies on animals complied with the *D6cret sur 1'Experimentation Animale* (French law on rules for animal experimentation; decree 87-848, 19 October 1987).

AcSDKP-NH_2 and AcSDKP were co-injected into mice at the dose of 1.0 mg/kg as an intravenous bolus (via the tail vein). Animals were anesthetized by an i.p. injection of pentobarbital 60 mg/kg. Blood was collected by abdominal vein puncture at T_0, T_5, T_{15}, T_{30}, T_{45}, T_{60}, T_{90}, T_{120} (min). A T_{180} sampling time was added for AcSDKP-NH_2. The ACE inhibitor, lisinopril (10^{-5}M), was added to each blood sample in order to prevent AcSDKP degradation by ACE. Five mice were used at each time-point. Blood was centrifuged at 4°C to obtain plasma and all samples were stored at –30°C

until analysis. At selected times (T_0, T_{15}, T_{60}, and T_{120}), tissues (spleen, liver, lung, kidney, and bone marrow) were collected and immediately frozen until extraction.

Sections of 200 to 400 mg of tissues were homogenized (Polytron, Kinematica GmBH, Littau, Switzerland) in the extraction solvent (2M acetic acid). The homogenates were centrifuged at 4500 rpm for 15 min at 4°C. The supernatants were diluted in the extraction solvent to obtain a 50 mg/mL tissue solution, which was stored at –20°C until assay. Bone marrow was collected by flushing the centro-medullary femur cavity with 500 μL saline. The samples were stored at –20°C.

Before assay, bone marrow samples were sonicated for 20 seconds, treated with methanol (1 mL methanol for 0.2 mL sample) and centrifuged at 4500 rpm for 15 minutes at 4°C. Methanolic solutions from supernatants of bone marrow and tissue acetic extracts were evaporated to dryness and resuspended either in human plasma (ETS, Rungis, France) for AcSDKP-NH_2 measurement or in EIA buffer (0.1 M phosphate buffer pH 7.4 , 0.15 M NaCl, 0.1 % BSA, 10^{-3} M EDTA and 0.01% sodium azide) for AcSDKP detennination.

Pharmacokinetic and Statistical Analysis

Pharmacokinetic analysis was performed using Siphar software (Simed, Cr&tell, France). The plasma concentration-time profiles for AcSDKP-NH_2 and AcSDKP were analyzed by a compartmental method. Endogenous plasma AcSDKP levels were substracted prior to pharmacokinetic analysis. The peak plasma concentration (Cmax) was obtained from experimental data. The area under the plasma-time curve from zero to infinity (AUC_{inf}) was determined by phannacokinetic modeling.

As very high plasma AcSDKP-NH_2 and AcSDKP concentrations were achieved in the pharmacokinetic study, the tissue concentrations for spleen, kidneys, lungs, and liver have been corrected for blood contamination according to the following formula:

$$C_c = (C - f^* C_b)/(1 - f)$$

where C_c is the tissue concentration corrected for blood contamination, C_b is the blood concentration, C is the total tissue concentration and f is the proportion of total blood volume in spleen (0.20), kidneys (0.24), lungs (0.30), and liver (0.25).(21)

Statistical analysis was performed using Sigmastat software (Jandel Corporation, San Rafael, CA). Tissue AcSDKP levels were analyzed by one-way ANOVA for the time effect, in order to distinguish endogenous

from administered AcSDKP. The assumptions of ANOVA (homogeneity of variances and normality) were checked for each variable and natural logarithmic transformation was applied where appropriate. If the F test was significant ($p < 0.05$), pairwise comparisons with the T_o group were performed using the Student-Newman-Keuls method.

RESULTS

Assay Development

Control of Immunogen by MALDI-TOF Mass Spectrometry

MALDI-TOF mass spectrometry analysis was performed on samples containing BSA alone or coupled to AcSDKP-NH_2. Masses were found at 66523.8 and 73545.4 for uncoupled BSA and for BSA coupled to AcSDKP-NH_2, respectively. The mass difference (7021.6) showed 12 molecules of AcSDKP-NH_2 bound per molecule of BSA, indicating a coupling efficiency of 56

Antiserum Selection

Antiserurn selection was performed according to the criteria of the lowest cross-reactivity toward AcSDKP and the best sensitivity IC_{50} for AcSDKP-NH_2. All three rabbits gave satisfactory results since the first bleedings exhibited an IC_{50} for AcSDKP-NH_2 in the 20 to 40 nM range, with cross-reaction coefficients less than 0.01%. At the end of the immunization protocol, the selected bleeding was L1550 S7 with IC_{50} for AcSDKP-NH_2 of 1.4 nM and no cross-reaction toward AcSDKP. After optimization of reagent concentrations, the selected activity of the enzymatic tracer for use in the enzyme immunoassay was 0.5 Ellman unit and the bleeding was diluted 1:500,000.

Matrix Interferences

In order to assess AcSDKP-NH_2 in mouse plasma, we tested two different approaches to sample handling: dilution in a reference matrix and liquid-liquid extraction (methanol). The optimal results (based on analytical recovery) were obtained by diluting mouse plasma samples (at least four-fold) in human plasma (ETS, Rungis, France).

For tissue samples, acetic extracts were evaporated to dryness and resuspended in human plasma. The recovery of the extraction procedure, evaluated by spiking tissue extracts with known amounts of AcSDKP-NH_2, was in the range of 80-120%. The elimination of matrix interferences required a five-fold dilution of blank tissue extracts in human plasma.

Assay Validation

Figure 2 depicts a typical standard curve of AcSDKP-NH_2 in human plasma. The range of reliable response covers AcSDKP-NH_2 concentrations from 0.32 to 20 nM. The IC_{50} values ranged from 1. 8 to 2.2 nM in 10 separate experiments.

The specificity of the assay was evaluated by a cross-reaction study. AcSDKP, potential degradation products, and structural analogs were tested. As shown in Table 1, the selected antiserum (L1550 S7) exhibits a high specificity since it did not cross-react with the tested compounds.

The limit of quantitation (LOQ) was established by analyzing 8 individual mouse plasmas spiked with AcSDKP-NH_2 at 0.5, 1 and 2 nM. The lowest concentration of AcSDKP-NH_2 allowing good assay precision (CV less than 20%) and acceptable accuracy (accuracy ratio in the range of 85 to 115%) was found to be 1 nM. For tissues, the LOQ was 100 pmole/g since 2.5 mg of tissue were processed in our extraction protocol (50 μL of a tissue extract at 50 mg/mL evaporated to dryness and resuspended in 250 μL human plasma).

The stability study was designed to confirm the absence of any AcSDKP-NH_2 hydrolysis by ACE or by other peptidases in mouse plasma, and to evaluate the stability of AcSDKP-NH_2 under assay conditions. Recoveries of AcSDKP-NH_2 spiked in mouse plasma and in tissue acetic extract were within 15% of their initial values, thus demonstrating the stability of AcSDKP-NH_2 in plasma and in tissue acetic extracts at 4, 20, and 37°C. Futhermore, no alterations were detected after 3 freeze-thaw cycles.

For accuracy and precision determination, quality control QC samples were prepared by spiking AcSDKP-NH_2 in mouse plasma and in an acetic extract of liver. The results are summarized in Table 2. In the within-run (intra-day) experiments, the CVs (precision) obtained for plasma and tissue QC samples ranged from 1.4 to 7.8% and the accuracy, expressed as analytical recovery (found / theoritical concentration) ranged from 97 to 111%. In the between-run experiments, the CVs and the recoveries ranged from 1.4 to 20% and from 87 to 103%, respectively.

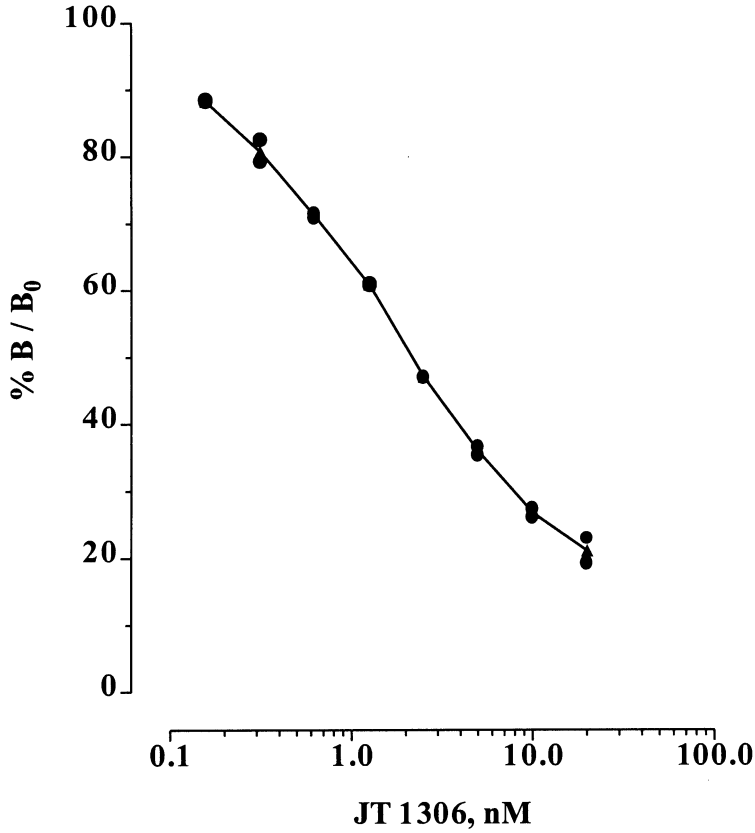

Figure 2. Typical standard calibration curve for AcSDKP-NH_2. Displacement of the enzymatic tracer by increasing amounts of AcSDKP-NH_2. B/B_0 values represent the ratio of tracer bound to the antibodies in the presence of (B) or absence of (B_0) of unlabeled AcSDKP-NH_2. Each standard was performed in duplicate.

Pharmacokinetic Study

An exploratory pharmacokinetic study was designed in mice in order to compare the plasma and tissue concentrations of AcSDKP-NH_2 to those of AcSDKP after co-administration of the two peptides by intravenous bolus.

Time-courses for plasma AcSDKP-NH_2 and AcSDKP concentrations are presented in Figure 3. Following intravenous administration, the time-courses of plasma AcSDKP-NH_2 and AcSDKP levels were best fitted by using a two compartment approach. Figure 3 shows that the plasma

Table 1. Cross-Reactivity Study

Cross-Reactant	CR (%) [a]
Acetyl-Ser-Asp-Lys-Pro-NH_2	100
Acetyl-Ser-Asp-Lys-Pro-OH (AcSDKP)	0.001
H-Ser-Asp-Lys-Pro-NH_2	0.17
H-Asp-Lys-Pro-NH_2	0.0001
H-Lys-Pro-NH_2	0.0005
Acetyl-Ser-Asp-OH	< 0.0001
Acetyl-Ser-Asp-Lys-OH	0.0025
Acetyl-Ala-Asp-Lys-Pro (AcADKP)	< 0.0001
Lisinopril	< 0.0001

[a] Cross-reactivity was determined by comparing the molar IC_{50} of each peptide to that of AcSDKP-NH_2 and expressing it as a percentage of AcSDKP-NH_2 immunoreactivity.

Table 2. Accuracy and Precision of the EIA Method for the Determination of AcSDKP-NH_2 in Mouse Plasma and Tissues

		Plasma (nM)			Tissue[a] (nmol/g)	
Parameter		10.0	2.0	1.0	1000	500
Intra–Day[b]	Precision (CV,%)	1.4	6.0	7.8	3.9	7.3
	Accuracy (%)	103	111	109	97	97
Inter–Day[c]	Precision (CV,%)	13	12	18	20	11
	Accuracy (%)	103	93	92	87	91

[a] Liver.
[b] n = 5 for plasma and tissue.
[c] n = 6 for plasma and n = 5 for tissue.

concentrations measured for AcSDKP-NH_2 are higher than those of AcSDKP administered at the same dose, and that AcSDKP is more rapidly cleared from the plasma than AcSDKP-NH_2. The maximal plasma concentrations (C_{max}) which corresponds to the first sampling time, and the plasma AUC_{inf} (290958 and 11198 nM.min for AcSDKP-NH_2 and AcSDKP, respectively) were increased 10- and 26-fold, respectively, for AcSDKP-NH_2 when compared to AcSDKP.

Tissue concentrations of AcSDKP-NH_2 and AcSDKP are presented in Table 3. After the elimination of blood contamination,(21) AcSDKP-NH_2 was only found in kidney and liver at T_{15}, although contamination by

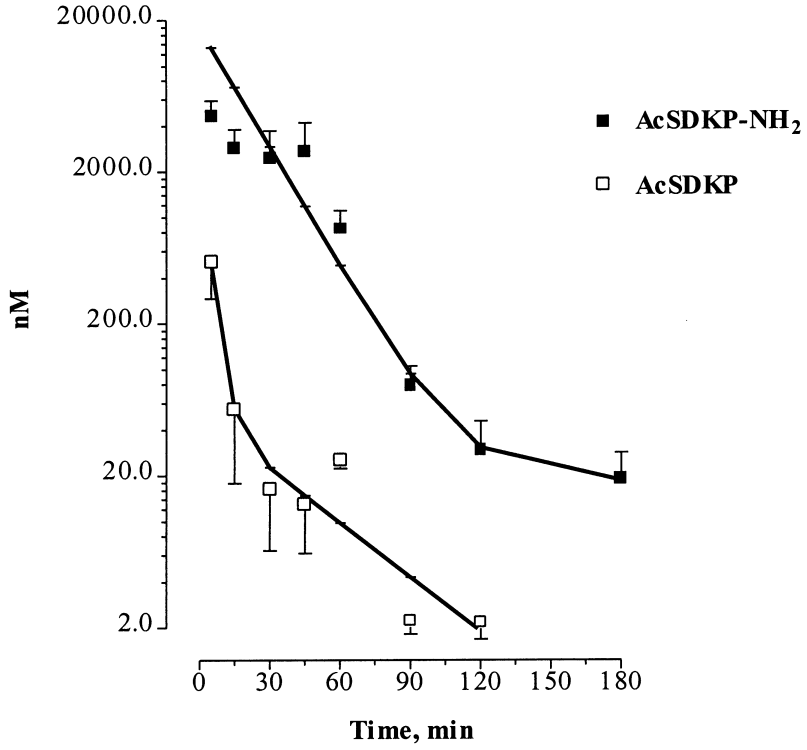

Figure 3. Time courses for AcSDKP-NH_2 and AcSDKP concentrations in plasma. Mice were co-injected (intravenous bolus of 1mg/kg) with AcSDKP-NH_2 and AcSDKP. Values are mean ± SD (n = 5). Endogenous AcSDKP levels (0.69 ± 0.36 nM) were subtracted for the determination of pharmacokinetc parameters. Lines represent the modelized pharmacokinetic profiles.

AcSDKP-NH_2 in urine remains difficult to exclude for the kidney. In contrast, a statistically significant increase in AcSDKP was found in spleen (at T_{15}), kidney (at T_{15} and T_{30}, lung (at T_{15}) and liver (at all times). The two compounds were found in femoral bone marrow at all times, with AcSDKP levels 8-fold higher than those of AcSDKP-NH_2 at T_{15}.

DISCUSSION

The tetrapeptide AcSDKP, a physiological regulator of hematopoiesis, prevents *in vitro* and *in vivo* the hematopoietic stem cells to enter into the S-

Table 3. Tissue AcSDKP and ACSDKP-NH_2 Levels

	AcSDKP				
	Spleen [a]	Kidney [a]	Lung [a]	Liver [a]	Bone Marrow
	pmol/g tissue				*pmol/mL*
T_0	850 ± 197	147 ± 42	454 ± 121	158 ± 18	1.94 ± 0.56
T_{15}	1239 ± 93 [b]	1157 ± 303 [b]	866 ± 191 [b]	644 ± 130 [b]	45.35 ± 23.17 [b]
T_{60}	1030 ± 252	874 ± 436 [b]	419 ± 67	423 ±106 [b]	8.56 ± 2.43 [b]
T_{120}	1041 ± 80	237 ± 29	489 ± 128	372 ± 9 [b]	14.82 ± 4.09[b]
	AcSDKP-NH_2				
T_{15}	ND [c]	168 ± 150	ND	138 ± 127	6.16 ± 2.02
T_{60}	ND	ND	ND	ND	2.54 ± 0.88
T_{120}	ND	ND	ND	ND	3.00 ± 0.74

[a] Values corrected for blood contamination (see materials and methods).
[b] $p < 0.05$ versus T_0 by Student-Newman-Keuls test.
[c] Not detectable.

phase of the cell cycle. Such inhibition of the proliferation has been shown to decrease the sensitivity of primitive hematopoietic cells to the toxicity of anticancer treatments.(22) Recently, AcSDKP has been shown to be a physiological substrate of ACE.(10, 11) The potential use of AcSDKP in human to reduce *in vivo* the damage of stem cell compartment resulting from treatment with chemotherapeutic agents or ionizing irradiation prompted the development of new AcSDKP derived molecules highly stable towards ACE degradation potency.(13) Four molecules were then synthesized by modifying the peptide bond or by modifying the C-terminus part of the peptide (AcSDKPNH_2). The biological efficacy of these analogs has recently been reported since all of them have been shown to inhibit *in vitro* the entry of murine hematopoietic precursors into the cell cycle.(14) In this context, the objectives of the present study were to develop and validate a sensitive and specific enzyme immunoassay for the measurement of AcSDKP-NH_2 in biological fluids in order to support pharmacokinetic and pharmacodynamic studies.

Since generated antibodies should discriminate between AcSDKP-NH_2 and AcSDKP and, therefore, should selectively recognize the amide part of AcSDKP-NH_2, two main strategies were available for the coupling: the use of glutaraldehyde, which selectively reacts with the free amine of the lysine residue (the amide function is not reactive), and the use of

carbodiimides which react with the free carboxyl group of the aspartate residue. We chose the glutaraldchyde method, known to be efficient with peptides containing a lysine residue.

Validation of the immunoassay showed that it is well adapted to pharmacokinetic studies, since it is robust, accurate, and precise (inter- and intra-assay coefficients of variation were all below 20%). The critical point to assess was the specificity of the assay, since endogenous AcSDKP has been reported in plasma (at the nanomolar level) and tissues such as spleen, bone marrow, lung, kidney, and brain where it appears to be 100- to 1,000-fold more concentrated than in plasma.(16) Assay specificity was confirmed by the cross-reaction study and by the absence of any immunoreactivity in blank plasma and tissue samples. The validation study also indicates that AcSDKP-NH_2 is stable in mouse plasma and that it is not hydrolyzed by *in vivo* ACE or by other enzyme, confirming previous *in vitro* stability studies that have been performed with rabbit lung ACE.(13)

The immunoassay was applied to a pharmacokinetic study to evaluate the potential therapeutic relevance of AcSDKP-NH_2. AcSDKP-NH_2 and AcSDKP were co-injected to mice at the dose of 1 mg/kg. Plasma concentrations and tissue distribution of AcSDKP-NH_2 and AcSDKP were then compared. The metabolism and elimination of AcSDKP have already been studied in healthy volunteers(23) and in rats.(20) These studies underlined that the elimination of the tetrapeptide is governed by two mechanisms: metabolic clearance by ACE and urinary elimination. In the case of AcSDKP-NH_2, the metabolic clearance is suppressed since this molecule is not hydrolyzed by ACE or other peptidases. Consequently, after administration, we showed that plasma AcSDKP-NH_2 levels were higher than those of AcSDKP, with AUC_{inf} and C_{max} values, respectively, 26- and 10-fold higher than that of AcSDKP. However, the elimination of AcSDKP-NH_2 from the plasma compartment remained relatively rapid, probably because of extensive renal filtration.

We also compared the tissular distribution of AcSDKP-NH_2 to that of AcSDKP. AcSDKP concentrations were significantly increased in all the evaluated mouse tissues at T_{15}. High AcSDKP levels were present until T_{60} in kidney and T_{120} in liver and bone marrow. However, increased AcSDKP concentrations in kidney should be interpreted with caution, because we cannot exclude the possibility of contamination by AcSDKP from urine, where it is abundant.(20) Owing to the strong structural analogy between AcSDKP and AcSDKP-NH_2, the same tissular penetration properties were expected for the two drugs. Unlike AcSDKP, AcSDKP-NH_2 exhibited very poor tissular distribution following its intravenous administration, since it was not found in lung or spleen at any time and was present in small amounts in kidney and liver only at T_{15}. Interestingly, however,

AcSDKP-NH_2 was found at all times in bone marrow, which could be one of the putative target organs for its pharmacological action.(24)

In conclusion, we have developed a sensitive and highly specific enzyme immunoassay for AcSDKP-NH_2 which does not cross-react with endogenous or administered AcSDKP. By means of this assay, which has been validated for pharmacokinetic and pharmacological studies, we have demonstrated that AcSDKP-NH_2 administered to mice is more slowly eliminated than AcSDKP. Taking into account that AcSDKP-NH_2 was shown previously to be a potent inhibitor of primitive hematopoietic cells proliferation *in vitro*,(14) this enzyme immunoassay can be used to *support in vivo* studies in order to test the efficacy of AcSDKP-NH_2 and to establish pharmacokinetic-pharmacodynamic relationships.

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