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Extraction of the synthetic lytic peptide, cecropin B, from biological fluid and analysis using reversed-phase high-performance liquid chromatography

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Abstract

The lytic peptide cecropin B, originally isolated from the giant silk moth *Hyalophora cecropia*, has been found to possess antibacterial and cell lysis properties in vitro and some anticancer activity in vivo. An HPLC method was developed to study synthetic cecropin B concentrations in biological fluids. Cecropin B was recovered from culture medium by solid-phase extraction (40.0 ± 2.4%), whereas in plasma it was highly protein-bound. The peptide was dissociated from proteins by citric acid and recovered by ultrafiltration (64.6 ± 5.9%) and was unstable in plasma (half-life, 0.57 ± 0.11 h). These analytical methods will facilitate future in vivo pharmacokinetic studies.

1. Introduction

Lytic peptides are increasingly being found to be involved in the antimicrobial defence system in a number of animal species, including insects [1]. Cecropins are one family of these peptides that were first isolated from the haemolymph of the giant silk moth, *Hyalophora cecropia* [1]. Cecropin B is 35 residues long with a relative molecular mass of 3835 and has a strongly basic N-terminus linked to a neutral C-terminus [2,3]. The overall structure, deduced by NMR for cecropin A, is two near-perfect, amphipathic segments joined by a flexible glycine–proline link [4,5]. A cecropin-like 31 residue peptide (cecropin P_1) has been isolated from the small intestine of the pig [6], suggesting that the cecropins may be widespread throughout the animal kingdom (Fig. 1).

Antibacterial activity has been reported for cecropins B and P_1 in vitro [7,6]. Cecropin B was also cytotoxic to a number of different mammalian cancer and non-cancer cell lines in vitro [8]. Preliminary studies have found cecropin B to have anticancer activity in vivo [8]. The mechanism of action of the cecropins is thought to involve channel formation in membranes and subsequent lysis [9]. In order to relate levels of the peptide in a biological environment to its biological activity, it was necessary to develop a

Cecropin B KWKVFKKIEKMGRNIRNGIVKAGPAIAVLGEAKAL-NH_2

Cecropin P_1 SWLSKTAKKLENSAKKRISEGIAIAIQGGPR

Fig. 1. Primary structures of cecropins B and P_1.

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reliable, sensitive assay for quantifying synthetic cecropin B concentrations in biological fluids. Preclinical pharmacokinetic studies of cecropin B have not previously been performed, and such studies would be essential for adequate evaluation of potential in vivo activity of the peptide. A method for analysis of cecropin A analogues by reversed-phase HPLC has previously been described [10]. However, a method for extraction of this group of peptides from plasma has not been published.

The aim of this study was to develop and validate a method for measuring synthetic cecropin B in biological fluids with sufficient sensitivity and specificity to determine in vitro stability and carry out in vivo pharmacokinetic studies.

2. Experimental

2.1. Chemicals

HPLC-grade acetonitrile (far UV), HPLC-grade trifluoroacetic acid (TFA) and perchloric acid were obtained from Fisons (Loughborough, UK). Water was triple-distilled. Sodium phosphate salts, sodium thiocyanate and urea were purchased from Sigma (Poole, UK). HPLC-grade citric acid and ammonium sulphate were purchased from BDH (Poole, UK). Tissue culture medium (RPMI 1640) was obtained from Life Technologies (Paisley, UK). Cecropin B was synthesised and supplied by Proteus Molecular Design (Macclesfield, UK), and cecropin P_1 was purchased from Peninsula Labs. (Merseyside, UK). Both peptides were checked for contaminants by HPLC and were >97% pure. Cecropins were dissolved in 0.1 *M* phosphate buffer, pH 6.4.

2.2. Chromatography

Peptide analysis was carried out by reversed-phase HPLC, based on a previously published method [10]. Separation was achieved with a LiChrosorb RP-8 column (250 × 4 mm I.D., 7 μm particle diameter) obtained from Merck (Poole, UK). The Waters HPLC system consisted of two pumps, Model 501 and Model 6000A, a Model 680 automated gradient controller, a Model 717 autosampler and a Model 484 absorbance detector, all obtained from Millipore (Watford, UK). Mobile phase A contained 950 ml of water, 50 ml of acetonitrile and 500 μl of TFA. Mobile phase B contained 650 ml of acetonitrile, 350 ml of water and 500 μl of TFA. A linear gradient ran from 30% to 75% B over 16 min at a flow-rate of 1.5 ml min^{-1}. A period equal to the run time was allowed for the system to re-equilibrate. The peptides were detected at a wavelength of 206 nm and peak areas were recorded using a Waters Model 745B data module. Samples were stored at 4°C in the autosampler before injection and an injection volume of 50 μl was used throughout.

2.3. Solid-phase extraction

In earlier in vitro studies, the cytotoxicity of cecropin B was investigated following different exposure times [8]. A method was developed for extracting cecropin B from tissue culture medium (RPMI 1640) plus foetal calf serum, in order to determine its stability. Acetonitrile (1 ml) and 1 ml of TFA (0.05%) were passed through a C_{18} Bond-Elut 50-mg cartridge obtained from Varian (Walton-on-Thames, UK). Culture medium (180 μl) was spiked with 20 μl of peptide (500 μ*M*, 1.92 mg ml^{-1}) and added to the cartridge. The cartridge was washed with 0.05% TFA and the peptide was eluted with 150 μl of acetonitrile. The recovery of cecropin B from culture medium was represented as the percentage of a control sample in buffer that had not been passed through a cartridge.

The same solid-phase extraction was repeated for plasma spiked with cecropin B; however, the peptide failed to be retained on the cartridge. In an attempt to optimise solid-phase extraction from plasma, a range of Bond-Elut cartridges (C_{18}, CN, PH, OH, CH, C_8, C_2) was prepared by the addition of 1 ml of acetonitrile and 2 ml of

citric acid (0.05 *M*, pH 1.8); citric acid was shown to dissociate cecropin B from plasma proteins (see later). A mixture of 285 μl of plasma, 15 μl of cecropin B (1 m*M*, 3.83 mg ml^{-1}) and 200 μl of citric acid (0.2 *M*, pH 1.5) was added to the cartridges. Cartridges were washed with 1 ml of citric acid (0.05 *M*, pH 1.8) before addition of 300 μl of acetonitrile.

2.4. Ultrafiltration

To investigate the extent of binding of cecropin B to plasma proteins, ultrafiltration cartridges were used. Murine plasma (95 μl) and 5 μl of cecropin B (1 m*M*, 3.83 mg ml^{-1}) were ultrafiltered in a centrifree micropartition system obtained from Amicon (Stonehouse, UK), which contained a hydrophilic YMT membrane with a 30 000 molecular mass cut-off. Centrifree tubes were spun for 15 min at 1500 *g* and 4°C. The effect of pH on dissociation of cecropin B from plasma protein was investigated. A 60-μl aliquot of 0.5 *M* phosphate buffer at pH 3, 5 or 7, 0.05% TFA (pH 2.0) or 0.2 *M* citric acid (pH 1.5) was added to 130 μl of murine plasma and 10 μl of cecropin B (1 m*M*, 3.83 mg ml^{-1}). Samples were ultrafiltered, and filtrates were analysed by HPLC.

Cecropin P_1 was selected as the internal standard because it was stable, had similar absorptive properties to cecropin B and could be extracted using the same procedure. The percentage recovery of cecropins B and P_1 from murine plasma, in the presence of citric acid, was determined. Citric acid was chosen as a dissociating agent because its weak acidic properties meant that it would not precipitate out the peptide during the extraction procedure. Murine plasma or buffer (130 μl) was spiked with 10 μl of cecropins B, P_1 or buffer before addition of 60 μl of citric acid (0.2 *M*, pH 1.5). Samples were ultrafiltered, and filtrates were analysed by HPLC. Cecropins B (10 μl) and P_1 (10 μl) were added to 180 μl of phosphate buffer and analysed by HPLC; these samples represented 100% controls, and recovery of filtered samples was calculated as a percentage of these controls.

A preliminary experiment was performed to validate the methodology for use in future in vivo pharmacokinetic studies. Cecropin B was injected into a mouse (intravenously) at a dose of 40 mg kg^{-1} and blood was taken 1.5 min later by cardiac puncture. The blood sample was collected into heparinised tubes and kept on ice before centrifugation at 1500 *g* for 10 min to separate the plasma. The internal standard and citric acid were added to the plasma before ultrafiltration.

Simple methods of protein precipitation were also tried in an attempt to extract cecropins from plasma. Plasma (90 μl), 10 μl of cecropin B (1 m*M*, 3.83 mg ml^{-1}) and 100 μl of 6 *M* urea (pH 9.7), 3 *M* sodium thiocyanate (pH 9.9), saturated ammonium sulphate (pH 4.8) or 10 μl of perchloric acid (4 *M*, pH < 1.0) plus 90 μl of TFA (0.05%, pH 2.0) were spun at 7500 *g* in a microcentrifuge for 2 min. The supernatant was analysed by HPLC.

2.5. Stability

In the ultrafiltration procedure, filtrates were stored at 4°C in citric acid in the autosampler before injection, therefore, stability of the peptides was determined under these conditions. Citric acid (0.2 *M*, pH 1.5) was spiked with cecropins B and P_1 (final concentration 25 μ*M*; 0.10 mg ml^{-1} and 0.08 mg ml^{-1}, respectively) and stored at 4°C in the autosampler. Injections were made every hour over a 22-h period.

The stability of cecropin B was determined at 37°C in culture medium and plasma. Culture medium was spiked with 50 μ*M* cecropin B (0.19 mg ml^{-1}) and incubated at 37°C. Samples (200 μl) were taken at regular intervals, and peptide was extracted using solid-phase extraction (detailed previously) before analysis by HPLC. Murine plasma (925 μl) and 75 μl of cecropin B (1 m*M*, 3.83 mg ml^{-1}) were incubated at 37°C and samples (100 μl) were taken at regular intervals and stored on ice. Cecropin P_1 (10 μl of 1 m*M*) and 40 μl of citric acid (0.2 *M*, pH 1.5) were added before samples were ultrafiltered and analysed by HPLC.

2.6. Calibration

Calibration curves were produced in triplicate over a range of cecropin B concentrations (0–50 μM) to ensure a linear relationship between peak area and concentration. Murine plasma (120 μl) was spiked with 20 μl of cecropin B or buffer and 10 μl of cecropin P_1. A 50-μl aliquot of citric acid (0.2 M, pH 1.5) was added before samples were ultrafiltered and analysed by HPLC. Graphs were plotted of the peak-area ratio of cecropin B to cecropin P_1 against cecropin B concentration.

3. Results

Cecropins B and P_1 were successfully analysed by HPLC at 206 nm as shown in Fig. 2a and b.

Synthetic cecropin B was extracted from culture medium using a C_{18} Bond-Elut cartridge. In culture medium, recovery of 50 μM (0.19 mg ml^{-1}) cecropin B was $40.0 \pm 2.4\%$ and its half-life at 37°C was 11.8 h. Less than 4% of cecropin B could be recovered from murine plasma using the same procedure, which is attributable to high protein binding ($>96\%$). This can be concluded from the observation that no cecropins could be detected in the ultrafiltrate of spiked plasma. The extent of protein binding in a spiked plasma sample was not altered by a change in pH (3, 5 or 7) or addition of 0.05% TFA. Only when 0.2 M citric acid (pH 1.5) was added to plasma spiked with cecropins, recovery was increased. The recoveries of 50 μM (0.16 mg ml^{-1}) cecropin P_1, 10 μM (0.04 mg ml^{-1}) and 50 μM (0.19 mg ml^{-1}) cecropin B from murine plasma were 52.7 ± 2.3, 63.4 ± 8.3 and $64.6 \pm 5.9\%$, respectively (Table 1). The peptide peaks were separated from interferent plasma peaks (Fig. 2c and d). The limit of UV detection at 206 nm for cecropin B was 2 μM (230 ng in column injection) after extraction from plasma. When cecropins, dissolved in buffer, were ultrafiltered, recovery was approximately 80% compared to control samples in the same buffer, that had not been filtered.

Through repetitive analyses of spiked murine plasma samples, the coefficient of variation (C.V.) for inter- and intra- assay reproducibility was found to be less than 10% (Table 2). There was a linear relationship between concentration (2.5–50 μM) of cecropin B and peak area ($r = 0.992 \pm 0.005$) in the plasma calibration samples. From triplicate determinations, each containing seven calibration points, the mean slope of the calibration line was 0.0263 ± 0.0016 and the mean y-intercept (ratio of cecropin B to cecropin P_1) was -0.0187 ± 0.0246.

Cecropins B and P_1 were stable in 0.2 M citric acid (pH 1.5) at 4°C, yielding approximately complete recovery even after 22 h of exposure. The stability of cecropin B at 37°C was measured in murine plasma and the mean half-life of cecropin B, determined from three independent experiments (Fig. 3), was 0.57 ± 0.11 h.

Citric acid was added to spiked plasma samples before addition to seven different types of Bond-Elut cartridges. In contrast to ultrafiltration, peptide recovery was less than 30% in most cases and in a few instances about 40%. In a similar way recoveries from plasma precipitation, with different precipitation agents, were substantially lower. Perchloric acid (4 M) yielded 29.2% recovery, whereas recoveries with urea (6 M), sodium thiocyanate (3 M) and saturated ammonium sulphate were less than 10%.

When cecropin B was injected intravenously into a mouse, the peptide could be detected in the plasma after 1.5 min (Fig. 4) at a concentration of 28.7 μM.

4. Discussion

An HPLC method was developed for detection of cecropin B, and procedures were successfully established for extracting the peptide from biological fluids. Using the solid-phase extraction procedure that was developed, the half-life of cecropin B in culture medium was 11.8 h. Previously, lytic activity of cecropin B against HRT-18 cells in vitro was found to be similar after exposure periods of 1, 24 and 96 h [8] and the half-life of the peptide may be one important contributing factor of this activity. Solid-phase

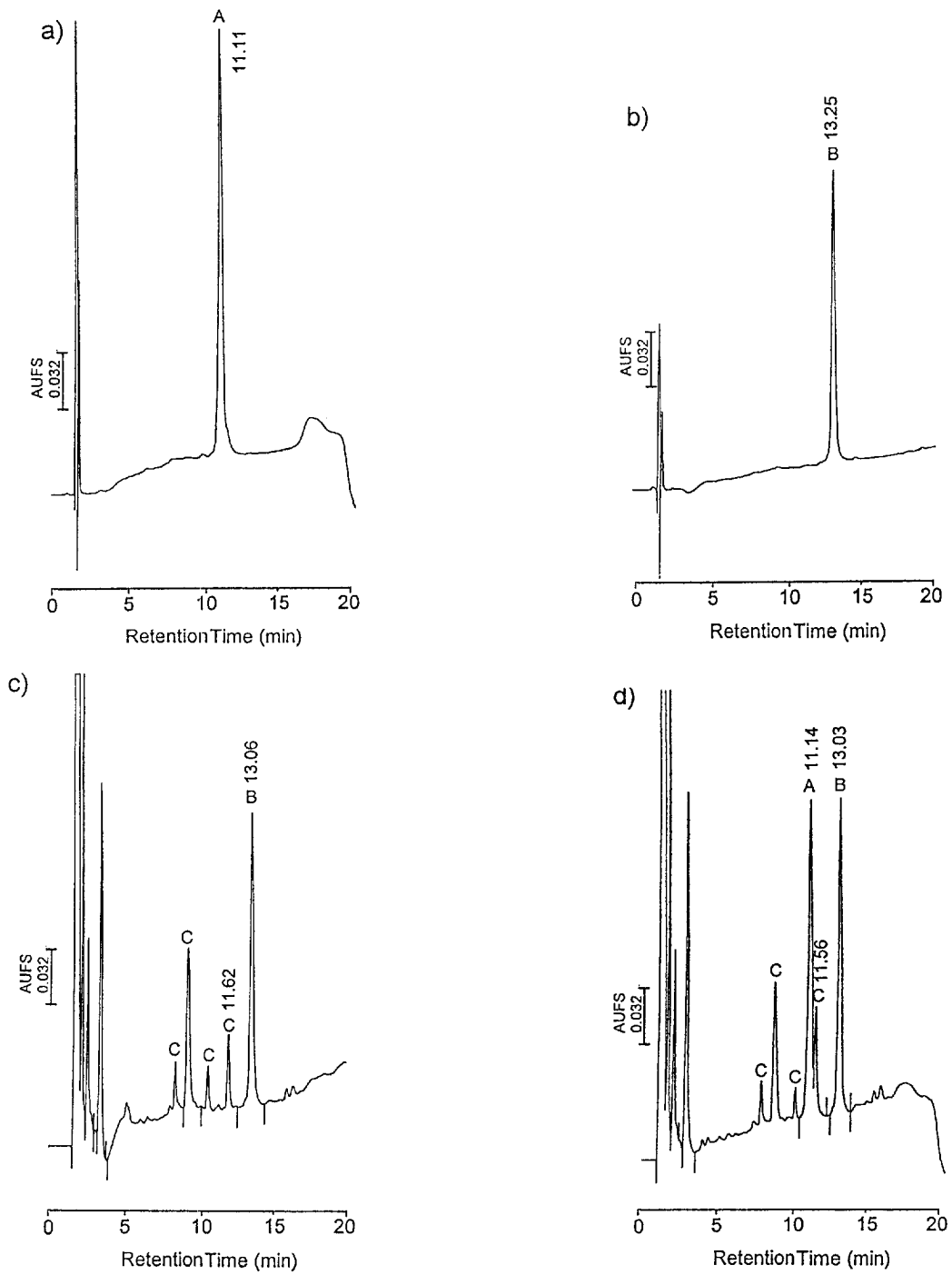

Fig. 2. Representative chromatograms of (a, b) reference standards containing 50 μM (0.19 mg ml^{-1}) cecropin B and 50 μM (0.16 mg ml^{-1}) cecropin P_1, respectively, in buffer, (c) murine plasma spiked with 50 μM (0.16 mg ml^{-1}) cecropin P_1 only and (d) murine plasma spiked with 50 μM (0.19 mg ml^{-1}) cecropin B and 50 μM (0.16 mg ml^{-1}) cecropin P_1. Peaks: A = cecropin B; B = cecropin P_1; C = interferences from plasma. Numbers adjacent to peaks show retention times (minutes).

Table 1
Recovery of cecropins after ultrafiltration

Sample	Recovery (mean ± S.D., $n = 4$) (%)
50 μM (0.19 mg ml^{-1}) cecropin B in buffer	78.8 ± 7.0
50 μM (0.16 mg ml^{-1}) cecropin P_1 in buffer	80.9 ± 6.7
10 μM (0.04 mg ml^{-1}) cecropin B in plasma	63.4 ± 8.3
50 μM (0.19 mg ml^{-1}) cecropin B in plasma	64.6 ± 5.9
50 μM (0.16 mg ml^{-1}) cecropin P_1 in plasma	52.7 ± 2.3

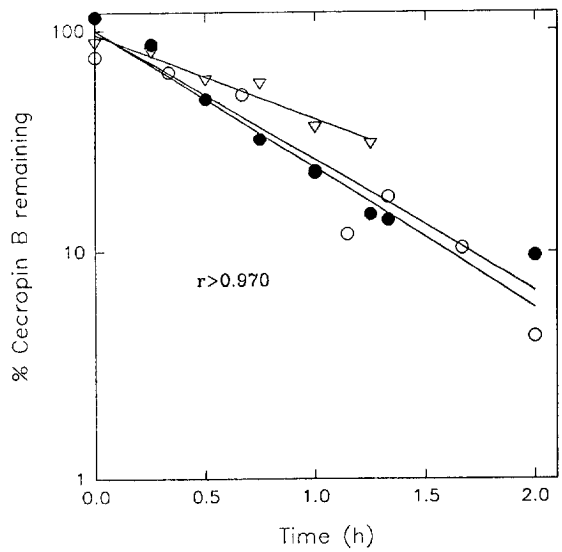

Fig. 3. Stability of cecropin B in murine plasma at 37°C. The curves were fitted by log-linear regression. The half-life of cecropin B in experiment 1 (○) is 0.48 h, in experiment 2 (●) 0.53 h and in experiment 3 (▽) 0.70 h.

extraction was investigated for isolation of cecropin B from plasma because this procedure is relatively inexpensive and allows the opportunity for automated sample preparation and concentration. The solid-phase extraction procedure used to extract cecropin B from culture medium proved unsuccessful in extracting the peptide from murine plasma, due to extensive protein binding of the peptide.

Cecropin P_1 was chosen as the internal standard because it is highly recoverable from plasma using the same procedure as for cecropin B and had similar chromatographic characteristics, which is attributable to corresponding hydrophobic properties. Using ultrafiltration, citric acid successfully dissociated the peptides from plasma, and recovery of cecropin B from plasma was adequate and yielded reproducible values of ca. 65%. Recovery of cecropins B and P_1 from buffer was ca. 80%, which suggested approximately 20% binding of the peptides to the filter in the cartridge. The cecropin B (35%) that was not recovered from plasma was probably due to binding to the filter and to incomplete dissociation from plasma proteins. The centrifree system was consistently more effective at recovering the peptides than several other ultrafilters from various manufacturers, including those with cellulose acetate or polysulphone membranes. The cecropins were stable in 0.2 M citric acid at 4°C for at least 22 h and was therefore chosen as the suitable solution for peptides to be stored in until analysis by HPLC.

Table 2
Within-day (intra-) and between-day (inter-) assay reproducibility in analysis for cecropin B in spiked murine plasma

Cecropin B concentration in plasma (μM)	Intra-assay variation		Inter-assay variation	
	Concentration determined (mean ± S.D., $n = 4$) (μM)	C.V.[a] (%)	Concentration determined (mean ± S.D., $n = 4$) (μM)	C.V.[a] (%)
10	11.3 ± 0.7	6.2	11.3 ± 0.8	7.1
50	50.1 ± 3.8	7.6	49.5 ± 3.5	7.1

[a] Coefficient of variation (C.V.) = (S.D./mean) × 100.

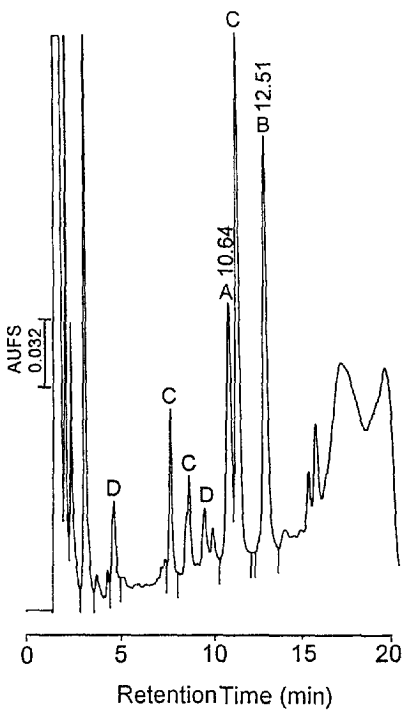

Fig. 4. Chromatogram of cecropin B in murine plasma following in vivo administration. Peaks: A = cecropin B; B = cecropin P_1; C = interferences from plasma; D = possible cecropin B metabolites. Numbers adjacent to peaks show retention times (minutes).

There was a linear relationship between the concentration of cecropin B extracted from murine plasma and peak area. Because cecropin B is a peptide it may have been expected to be susceptible to rapid breakdown in the plasma by peptidases within a few minutes. However, the half-life of the peptide was 0.57 h, suggesting it to be a possible candidate for future in vivo studies. The peptide had similar stability in whole murine blood (data not shown).

Attempts were made to develop an effective solid-phase extraction procedure by adding citric acid to peptide-spiked plasma samples prior to extraction. However, despite using several different types of Bond-Elut cartridges, there was low recovery of the peptides. Supporting experiments showed that the peptides were not retained on the Bond-Elut cartridges at this acidic pH. Peptides were retained, however, at pH 7.0, but on altering the pH to enhance binding to the solid-phase cartridge protein binding was increased; hence recovery was decreased. Several ion-exchange methods proved unsuccessful in extraction of cecropin B from plasma because the pH could not be readily adjusted without increasing the bound fraction of the peptide.

In conclusion, this study describes reproducible and sensitive methods to measure cecropin B in biological fluids. Cecropin B was also extracted from plasma after intravenous injection in a mouse, demonstrating that methods of extraction from plasma by ultrafiltration and UV detection on HPLC are suitable for future pharmacokinetic studies.

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