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Short communication

# Peptide quantification by matrix-assisted laser desorption ionisation time-of-flight mass spectrometry: Investigations of the cyclotide kalata B1 in biological fluids

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**Abstract**

A rapid method has been developed for the quantification of the prototypic cyclotide kalata B1 in water and plasma utilizing matrix-assisted laser desorption ionisation time-of-flight (MALDI-TOF) mass spectrometry. The unusual structure of the cyclotides means that they do not ionise as readily as linear peptides and as a result of their low ionisation efficiency, traditional LC/MS analyses were not able to reach the levels of detection required for the quantification of cyclotides in plasma for pharmacokinetic studies. MALDI-TOF-MS analysis showed linearity ($R^2 > 0.99$) in the concentration range 0.05–10 μg/mL with a limit of detection of 0.05 μg/mL (9 fmol) in plasma. This paper highlights the applicability of MALDI-TOF mass spectrometry for the rapid and sensitive quantification of peptides in biological samples without the need for extensive extraction procedures.


*Keywords:* Quantification; Cyclotides; kalata B1; Circular proteins; LC/MS; MALDI-TOF mass spectrometry

## 1. Introduction

The cyclotides [1] are a large family of head-to-tail macrocyclic peptides (∼30 amino acids in length) that have exceptional stability. The prototypic member of the family was first discovered on the basis of native medicine applications in Africa. A Red Cross worker observed women of the Lulua tribe utilizing the medicinal properties of a native plant *Oldenlandia affinis*, locally referred to as "kalata kalata" [2,3]. Effects of drinking a tea made from the leaves of the plant included the acceleration of uterine contractions and childbirth. The preparation by boiling and the oral administration suggested that the active ingredient was both bioavailable and thermally stable. The peptide kalata B1 (kB1) was isolated and noted to be responsible for the activity [3]. Initial attempts to sequence this unusual peptide proved unsuccessful owing to apparently blocked N- and C-termini. It was later discovered [4] that kalata B1 is just one member of a large family of peptides that contain a head-to-tail cyclized backbone and were named the cyclotides [1]. As a result of their unusual structures, they are resistant to sequencing using Edman degradation or tandem mass spectrometry [5]. As there are no termini and the molecules lack charged residues, the ionisation efficiency is lower than that of typical peptides of similar molecular weight and size.

Fig. 1 shows the structure of kalata B1 and its amino acid sequence. The cyclotides contain three disulfide bonds, which occupy the molecular core, resulting in the exclusion of hydrophobic residues, which instead form a surface-exposed hydrophobic patch that results in unusually long retention times when examined by reversed phase high-performance

*Abbreviations:* kB1, kalata B1; kB2, kalata B2; HPLC, high-performance liquid chromatography; LC/MS, liquid chromatography mass spectrometry; MALDI, matrix assisted laser desorption ionisation; TOF, time-of-flight

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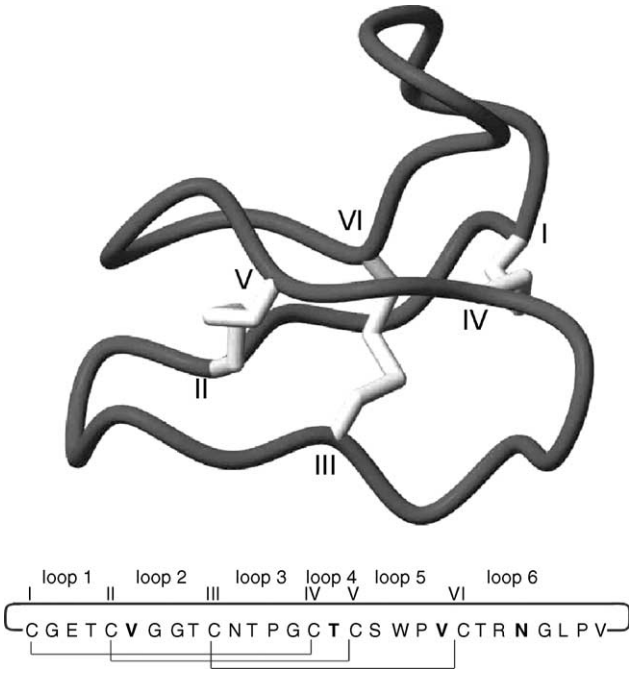


Fig. 1. Amino acid sequence and three-dimensional structure of kalata B1. The six conserved Cys residues are numbered with Roman numerals and the backbone loops between Cys residues are numbered 1–6. The structure of kalata B1 shows the cyclic cystine knot motif and the three disulfide bonds that occupy the core of the molecule. The internal standard kalata B2 differs from kalata B1 by five point mutations denoted in bold as follows: V6F, T16S, S18D, V21I and N25D.

liquid chromatography (HPLC). The disulfide bonds are arranged in a cyclic cystine knot motif [5,6]. In this motif an embedded ring in the structure, formed by two disulfide bonds and their connecting backbone segments, is penetrated by the third disulfide bond. Owing to this stable framework, the cyclotides are extremely resistant to enzymatic breakdown and are exciting candidates for drug design and development [4,7]. To utilize the cyclotide framework in medicinal applications, it is necessary to understand the pharmacokinetics of the parent molecules. We have already demonstrated their stability toward enzymes in the blood and digestive system [8], but processes such as drug elimination, excretion and metabolism need to be explored to assess the viability of a drug in vivo. To this end, a method for detecting and quantifying the cyclotides in plasma was required.

Since its introduction in 1988 [9,10], matrix-assisted laser desorption ionisation time-of-flight mass spectrometry (MALDI-TOF-MS) has been utilized extensively for the identification and molecular weight determination of a wide range of biological molecules, including natural products as well as synthetic molecules. MALDI has the ability to generate intact gas-phase ions of large, thermally labile biomolecules by desorption/ionisation from crystals comprised of a mixture of the matrix (small, volatile molecules) and the biomolecule. MALDI-TOF-MS has also become a valuable tool in peptide mapping and the sequencing of biopolymers [11]. Electrospray ionisation is susceptible to ion suppression effects, which render direct analysis of complex mixtures difficult as the presence of salts and other components can significantly decrease sensitivity. MALDI is extremely sensitive in being able to detect femtomole (fmol) levels of peptide. The MALDI process is more tolerant to the presence of buffers and salts and is able to analyse samples containing multiple biomolecular species.

The general application of MALDI for quantification was initially rejected owing to a number of factors. Significant variations in the ion signals resulting from consecutive laser shots at the same position within a spot, as well as from different positions on a given spot (known as “shot-to-shot” reproducibility) are seen, along with differences observed between sample spots (“sample-to-sample” reproducibility). These variations may be due to inhomogeneous co-crystallisation of matrix and sample molecules, or to fluctuations in laser power, and/or changes in detector response [12,13]. Recently, the application of MALDI for quantification has received attention as an alternative to current quantification methodologies. Applications include quantification of amino acids [14], lipids [15], natural products from food sources [16,17], small organics [13,18], antibiotics [19], oligonucleotides [20] and proteins and peptides [21–24].

The goal of the present study was to develop a robust method to allow the quantification of cyclotides in plasma for pharmacokinetic studies. To do so, it was necessary to investigate the potential of MALDI-TOF-MS for accurate, precise and reproducible quantification of macrocyclic peptides in rat plasma. This study describes a method for the accurate quantification of cyclotides within biological fluids with rapid and simple sample preparation.

## 2. Experimental

### 2.1. Materials and methods

#### 2.1.1. Extraction and purification of cyclotides

Native kalata B1 [5] and kalata B2 [25] were isolated from *O. affinis*. Fresh plant material (500 g) was ground and extracted with 50/50 (v/v) $CH_2Cl_2$/MeOH and the crude extract was partially purified by reversed phase flash chromatography, yielding a fraction containing predominantly cyclotides (5 g). This sample was purified further by preparative reversed phase HPLC to yield pure kalata B1 (∼125 mg) and kalata B2 (∼100 mg) as described previously [1].

#### 2.1.2. Standard calibration solution preparation

Peptide solutions were prepared by dissolving 1 mg of purified peptide in 1 mL of Milli Q $H_2O$ and accurately determining the concentration via UV analysis at 280 nm ($\varepsilon = 6050\ M^{-1}\ cm^{-1}$). The 50 μg/mL stock solutions of kalata B1 and kalata B2 were prepared in Milli Q $H_2O$. From these solutions, sets of 10 standard solutions containing 1 μg/mL kalata B2 as the internal standard and varying

amounts of kalata B1 (0.01–10 μg/mL) were prepared in either water or in plasma.

*2.1.3. Dosing of rats and sample analysis*

Rats were fasted overnight with free access to water. Anaesthetised rats were administered an intravenous (I.V.) dose of 3 mg/kg of peptide via the femoral vein over a 2 min period. Blood samples were obtained over a 3 h period. Approximately 300 μL of blood was collected from the tip of the tail into an eppendorff tube containing 30 μL of 500 IU/mL heparin. After centrifugation, 100 μL of serum was removed from each sample and stored at −20 °C until analysis.

Calibration solutions and plasma samples obtained from male Sprague–Dawley rats after intravenous injection dosing were treated as follows: 50–100 μL of plasma was taken and an appropriate amount of internal standard was added to yield 1 μg/mL kalata B2. A two-fold excess of acetonitrile was added to cause large proteins to precipitate out of solution. The solutions were then mixed and centrifuged at 14,000 rpm for 10 min. The supernatant was removed and used for mass spectrometric analysis.

*2.2. Instrumentation*

*2.2.1. Liquid chromatography/mass spectrometry (LC/MS)*

The standard calibration solutions and treated samples from the plasma stability studies were monitored by LC/MS on an HP 1100 Series HPLC (Agilent Technologies) coupled to a QStar Pulsar mass spectrometer (Applied Biosystems) equipped with an electrospray ionisation (ESI) source. Forty microliters of supernatant that had been diluted 1:1 in water following treatment was injected onto a Phenomenex C18 column (150 mm × 2.1 mm I.D., 5 μm) protected with a Phenomenex precolumn 0.5 μm filter utilizing a fast (20%) gradient and high (400 μL/min) flowrate at 60 °C column temperature. A gradient of 5–100% acetonitrile (0.1% formic acid) was utilized to elute the peptides, which were monitored by online electrospray mass spectrometry. The LC was directly interfaced to the mass spectrometer. The capillary voltage and cone voltage were 5300 and 30 V, respectively. Data acquisition and analysis were performed using the Analyst QS v1.0 software.

*2.2.2. Matrix-assisted laser desorption ionisation time-of-flight MS (MALDI-TOF-MS)*

0.5 μL of the supernatant was mixed with 0.5 μL of the matrix consisting of a saturated solution of α-cyano-4-hydroxycinnamic acid (CHCA) in 50% acetonitrile (0.05% formic acid) and mixed thoroughly. 0.5 μL of the mixture was spotted onto a 2 × 96 spot plate and was dried under a gentle stream of nitrogen gas at room temperature. All solutions were analyzed by MALDI-TOF mass spectrometry on a Voyager DE-STR mass spectrometer (Applied Biosystems). A spiral pattern for automated sample acquisition was used in which 20 positions on the MALDI spot were sampled and 100 shots per position were averaged giving 2000 shots total to yield representative spectra. The MALDI-TOF-MS was operated in positive ion reflector mode. The laser intensity was set to 2300, the accelerating voltage was set to 20,000 V; the grid voltage set to 64% of the accelerating voltage and the delay time was 165 ns. The low mass gate was set to 500 Da. Data were collected between 2500 and 3500 Da. Calibration was undertaken using a peptide mixture obtained from Sigma–Aldrich (MSCal1). The mixture contained bradykinin, $P_{14}R$, ACTH fragment 18–39 and insulin B chain. Data acquisition was performed using Voyager Instrument Control Panel v5.10 and analysed using Voyager Data Explorer v4.0 software.

*2.3. Statistics and method validation*

Calibration curves were constructed from the peak area ratios (LC/MS) or summed peak area ratios (MALDI) of the analyte to the internal standard versus the theoretical concentrations. Linear regression analysis of the standard curves and calculation of peptide concentration in rat plasma samples was undertaken using GraphPad Prism. Unknown sample concentrations were calculated using linear equations ($y = mx + b$) fitted for the calibration curves, where $y$ is the relative area (analyte/IS) and $x$ is the concentration of analyte. The precision of the method was determined by replicate analyses ($n = 3$) of plasma samples containing kalata B1 at different concentrations within the calibration range and presented as the standard deviation (SD). The accuracy of the method was expressed as (mean calculated concentration)/(spiked concentration) × 100. The limit of detection (LOD) was defined as the lowest sample concentration that could be detected (S/N > 3). The recovery of kalata B1 with its internal standard kalata B2 was determined by comparing the relative peak area of the analyte/IS extracted from plasma to those of standards prepared in water. The results were obtained from a range of concentrations and the averages were taken as mean recoveries.

## 3. Results and discussion

Existing methods utilized for the quantification of peptides in biological fluids are typically time consuming and require extensive sample preparation and in the case of macrocyclic peptides such as the cyclotides are limited by ionisation efficiency. MALDI-TOF-MS provides a direct method for analysis of complex mixtures and shows improved sensitivity over LC/MS analysis (9 fmol compared to 7 pmol). In the past it has not been favoured for quantification applications but the approach developed here shows that MALDI is very suitable for the quantitation of peptides in biological fluids. In the current study the prototypic cyclotide, kalata B1, was used as the analyte and kalata B2 was selected as the internal standard.

### 3.1. Preparation of calibration curves

A series of standards were prepared by spiking plasma with kalata B1 and adding a known amount of internal standard, kalata B2, followed by protein precipitation using a two-fold excess of organic solvent (acetonitrile) and centrifugation. Kalata B2 (MW = 2954) was selected as the internal standard as it has similar chemical properties but is distinct from kalata B1 (MW = 2890) in both retention time by HPLC and mass. Kalata B2 contains five point mutations, as shown in Fig. 1, resulting in the peptide being more hydrophobic than kalata B1. Initially, the calibration solutions were analysed by LC/MS using a C18 reversed phase column. A 1:1 dilution of the supernatant yielded a final acetonitrile percentage of ~30%, which was low enough to ensure that the peptides were retained on the column. Ions of interest (doubly-protonated ions) were extracted using the software and the resulting peaks were integrated for peak intensity and area.

Fig. 2 shows the chromatograms and electrospray mass spectra of the 1 μg/mL standard. A number of other peptides were also present in the plasma, as shown in the total ion chromatogram (Fig. 2A). Fig. 2B and C show the ion-extracted chromatograms for the analyte, kalata B1 (*m/z* 1446–1449) and the internal standard, kalata B2 (*m/z* 1477–1480). Single peaks with retention times of 7.20 and 7.67 min, respectively show the presence of these species within the sample. The mass spectra resulting from the average of all scans of the ion extracted peaks are shown as insets to Fig. 2B and C. Fig. 2D shows the calibration curve achieved using LC/MS. Assays were linear ($R^2 > 0.99$) in the concentration range 0.5–25 μg/mL. However, the LC/MS method could only provide a limit of detection (LOD) of 0.5 μg/mL (6.9 pmol) in plasma, which was not sensitive enough for in vivo pharmacokinetic studies, where an LOD of 0.05 μg/mL was required. Therefore, an alternate method for analysis was required and MALDI-TOF-MS was investigated for its use in quantification.

Fig. 3A shows the mass spectrum resulting from MALDI analysis of the 1 μg/mL standard. Fig. 3B shows the MALDI mass spectrum resulting from the analysis of a plasma blank, i.e. containing no analyte material, while Fig. 3C shows the MALDI mass spectrum of the lowest concentration (0.05 μg/mL; 8.7 fmol) plasma standard able to be analysed via this method. The signal-to-noise (S/N) ratio is approximately 3:1. Below this concentration, although observable, the analyte material was not significantly distinguishable from the background noise. In all samples, the $^{13}C$ isotopes are clearly resolved. The areas of the first five isotope peaks for the species of interest were summed and compared to the summed areas of the internal standard peaks to yield the calibration curve as shown in Fig. 3D. Each datapoint represents the mean value from three replicates and standard deviations for the measurements are indicated by the error bars. Calibration curves were also prepared using measurement of the maximum signal intensity (not shown). Linear

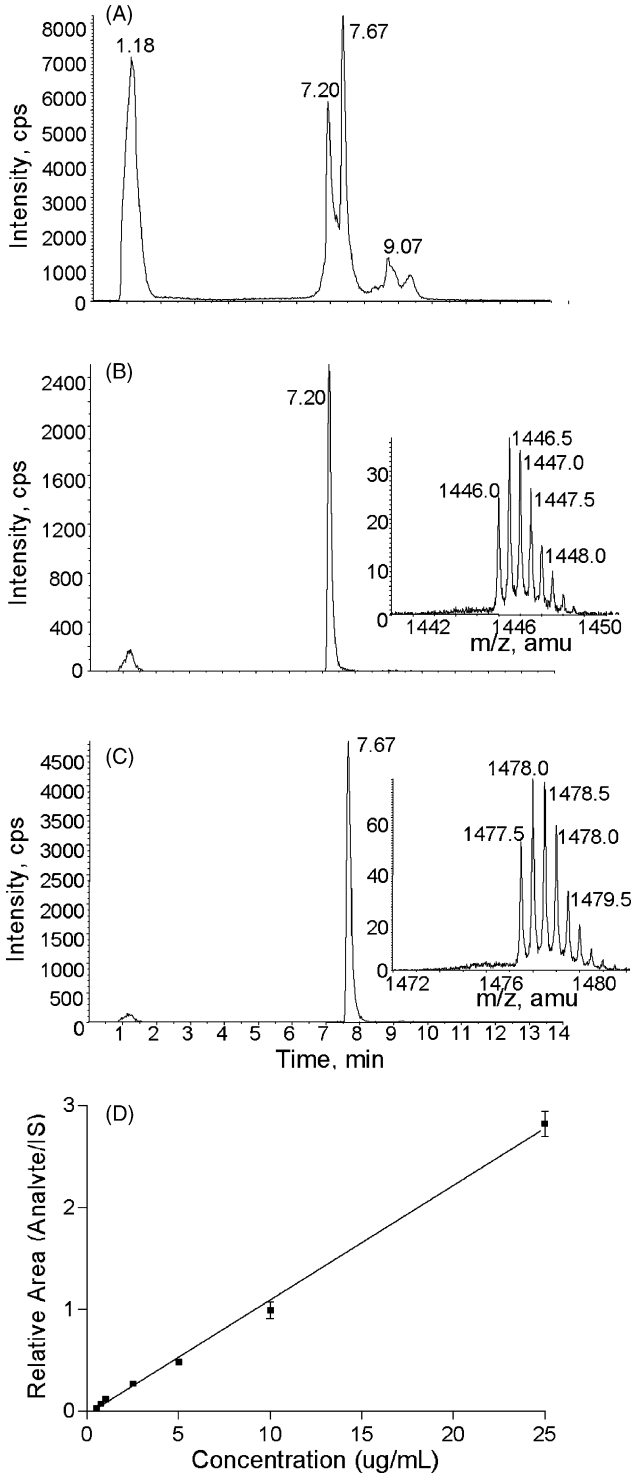


Fig. 2. LC/MS analysis of the standard calibration solutions: total ion chromatogram of the 1 μg/mL standard (A) and the individual ion-extracted chromatograms and electrospray ionisation mass spectra for the kalata B1 analyte (*m/z* 1446–1449, B) and for the kalata B2 internal standard (*m/z* 1477–1480, C). The LC/MS calibration curve for the plasma standards between 0.5 and 25 μg/ml (D).

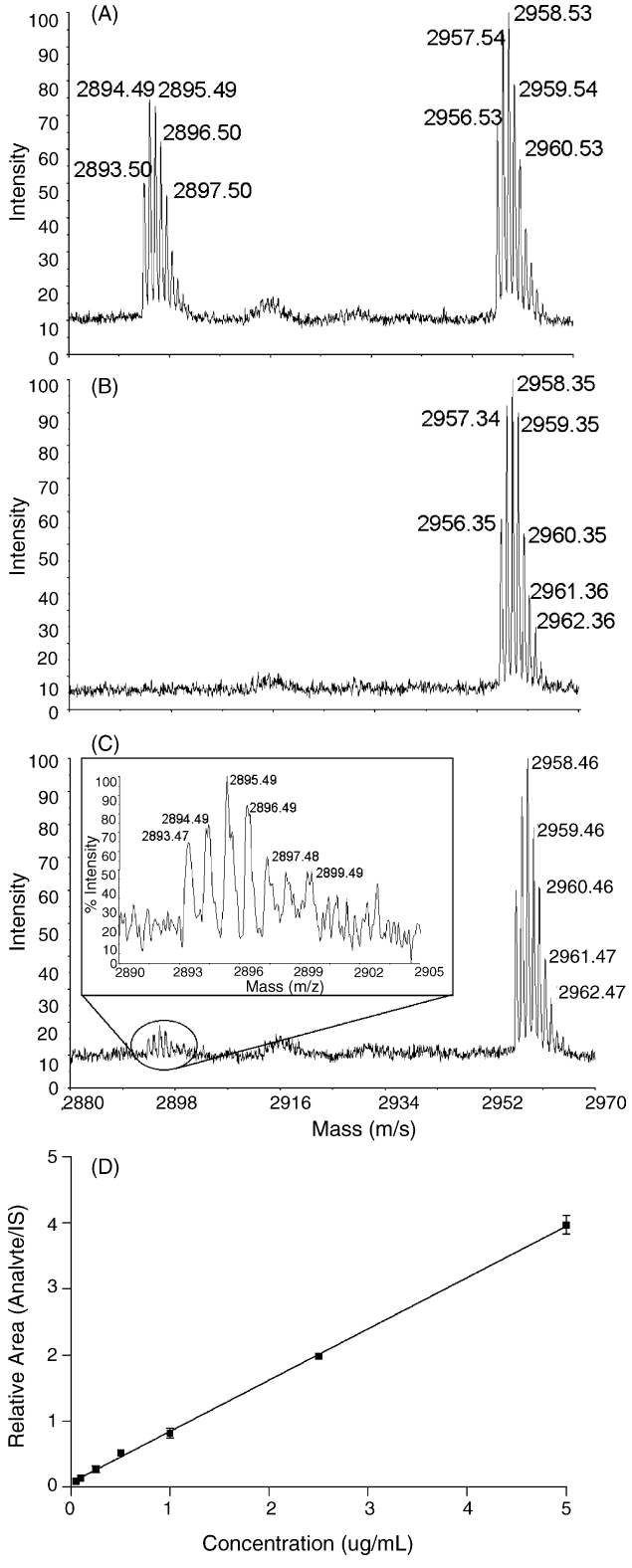


Fig. 3. MALDI-TOF mass spectrum from the analysis of: the 1 μg/mL standard calibration solution (A); a treated plasma blank showing only internal standard (B); and the 0.05 μg/ml standard showing a signal-to-noise ratio of >3:1 (C). The MALDI-TOF calibration curve for kalata B1 (D) using the relative sum of the peak area of kB1 over kB2.

regression analysis of peak area and signal intensity revealed a linear relationship over two orders of magnitude between 0.05 and 10 μg/mL, resulting in an $R^2$ of 0.9995 for peak area ratios and 0.9931 for signal intensity ratios. Above concentrations of 10 μg/mL, detector saturation was observed and the samples did not behave linearly. Dilution of the samples containing high concentrations of analyte overcame the problems with detector saturation and these samples then gave a linear response.

### 3.2. Method development and optimisation

The robustness of the protein precipitation step was assessed by measuring the recovery of peptides in plasma compared to water. The standards were prepared and treated in an identical manner to the plasma standards. Six concentrations ranging between 0.5 and 10 μg/mL within the LC/MS calibration curve were analysed. Recovery was between 93 and 130% for the six concentrations tested, with an average recovery of 112 ± 15%. The protein precipitation step was sufficient to remove most of the larger proteins while leaving the analyte and internal standard in solution.

Internal standards are usually applied to compensate for systematic and statistical errors during analysis. The use of an internal standard usually improves the accuracy of calibration curves and the use of isotopically labelled analogues of the analytes has provided the most precise results for small compounds, with correlation coefficients better than 0.95 [26–28]. These compounds should be chemically very similar to the analytes and for this reason kalata B2 was used as the internal standard and was used consistently at the same concentration (∼0.34 μM; ∼170 fmol).

The MALDI data acquisition method employed in which 20 positions on each target spot were summed and averaged to yield representative spectra gave excellent linearity over the 0.05–10 μg/mL concentration range with femtomole sensitivity. This method eliminates problems associated with signal degradation that have been shown to occur over time [29]. Standard curves prepared gave $R^2$ values >0.97 for each individual curve and when prepared using the average of triplicate measurements gave an $R^2$ of >0.99.

Recent developments in sample preparation techniques, such as the use of fast evaporation surfaces, have led to greater mass accuracy and improved resolution [30]. Many variations in sample preparation techniques have been examined to produce uniform crystal layers of homogeneous co-crystals. Multicomponent matrices were investigated and reported to supply uniform crystal types resulting in improved spectral reproducibility [29,31]. In the current study, a range of different sample preparation conditions was investigated to obtain uniform crystal layers of homogeneous co-crystals. Three matrix solutions were assessed: CHCA, sinapinic acid (SA) and 2,5-dihydroxybenzoic acid (DHB). The first of these was determined to be optimal for the cyclotide samples (data not shown) based on improved sensitivity, uniformity of crystal coverage of target area, resolution and mass accuracy of peaks

of interest. Pre-mixing of the plasma solutions with the matrix prior to spotting and accelerated drying under a gentle stream of nitrogen gas was observed to improve the crystallization homogeneity as seen under a microscope (data not shown). The fast evaporation technique has been used to generate uniform crystals and is based on the use of volatile solvents such as acetone, ethanol or methanol, that are evaporated by gentle blowing after application [31,32]. Alternatively, rapid removal of the solvent can be induced by a stream of high-purity nitrogen ('accelerated drying') [29] or under vacuum [19]. In both of these methods, the solution of sample and matrix are mixed and moved during the drying process, leading to small, evenly distributed crystals. In this work, it was found that matrix selection and application methods were critical to the production of uniform co-crystals and that the data acquisition method overcame problems of poor spot-to-spot reproducibility and signal degradation.

### 3.3. Application: intravenous dosing of rats

Using this technique, the amount of kalata B1 present in the blood after intravenous dosing of male Sprague–Dawley rats was determined at several time points allowing the generation of elimination curves for the prototypic cyclotide, kalata B1. The signal response area ratios of analyte to internal standard for the samples from the intravenous dosing of the rats were converted to concentrations using the equations derived from the spiked standard concentration curves. Fig. 4 shows the curve for the amount of kalata B1 detected in the blood after intravenous dosing at 3 mg/kg. The level increases dramatically over the first 5 min and then decreases in a roughly exponential manner as the peptide is degraded or excreted. The urine of the rats was analysed for the presence of kalata B1 or degradation products. A single peak corresponding to unmodified kalata B1 was observed. The elimination half-life in blood was determined to be approximately 33 min from analysis of the linear portion of the plot of the logarithm of the peptide concentration versus time.

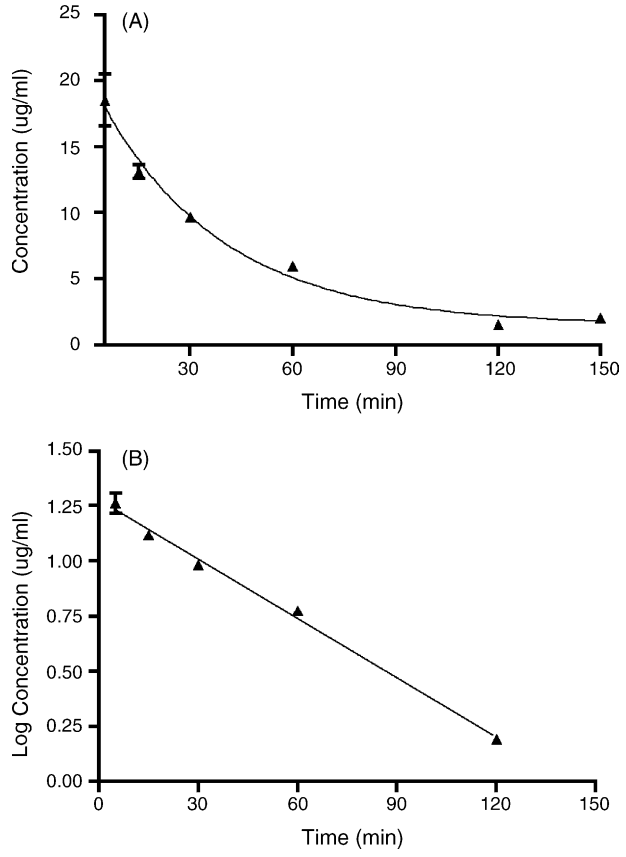


Fig. 4. Elimination curve following intravenous dosing of male Sprague–Dawley rats at 3 mg/kg (A) and log scale for calculation of elimination half-life from equation: $\log[C] = -0.009001t + 1.280$; $R^2 = 0.9866$ (B).

## 4. Conclusions

This study has demonstrated the applicability of MALDI-TOF-MS to the rapid, precise and sensitive quantification of peptides in plasma. This method differs from previously described approaches in that a unique data acquisition method has been utilized, which overcomes problems associated with poor spot-to-spot reproducibility and signal degradation. The dynamic range of the calibration curves extended at least two orders of magnitude with detection limits of 0.05 μg/mL (9 fmol) compared to 0.5 μg/mL (7 pmol) for LC/MS analyses of plasma standards. In addition to the greater sensitivity achieved, the analysis time was decreased 10-fold. This method has been applied to the analysis of plasma samples from pharmacokinetic studies.

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