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Improved method for quantitative analysis of the cyclotide kalata B1 in plasma and brain homogenate

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Abstract

This study provides a new method for quantifying the cyclotide kalata B1 in both plasma and brain homogenate. Cyclotides are ultra-stable peptides with three disulfide bonds that are interesting from a drug development perspective as they can be used as scaffolds. In this study we describe a new validated LC-MS/MS method with high sensitivity and specificity for kalata B1. The limit of quantification was 2 ng/mL in plasma and 5 ng/gmL in brain homogenate. The method was linear in the range 2–10,000 ng/mL for plasma and 5–2000 ng/g for brain. Liquid Chromatographic separation was performed on a HyPurity C18 column, 50 × 4.6 mm, 3 μm particle size. The method had inter- and intra-day precision and accuracy levels <15% and 12% respectively. Applying the method to *in vivo* plasma samples and brain homogenate samples from equilibrium dialysis yielded satisfying results and was able to describe the plasma pharmacokinetics and brain tissue binding of kalata B1. The described method is quick, reproducible and well suited to quantifying kalata B1 in biological matrices.

KEYWORDS

brain, cyclotides, kalata B1, liquid chromatography, mass spectrometry, pharmacokinetics

1 | INTRODUCTION

Kalata B1 is the archetypical cyclotide originally discovered in the plant *Oldenlandia affinis*.[1] It has garnered interest due to its high stability and potential use as a scaffold in drug development.[2,3] This family of peptides is defined by the cyclic cystine knot motif, comprised of a head to tail circular backbone and three disulfide bonds that is responsible for the high stability.[4] They appear to play a role in plant defence, not the least demonstrated by the insecticidal effect of kalata B1.[5,6] The cyclotides can be found in a number of plant families, including *Violaceae, Rubiaceae* and *Fabaceae,* with reports of large numbers of cyclotides found in single plant species.[7–9] Kalata B1 has shown bioactivity in several *in vitro* assays including, but not limited to, nematocidal activity, haemolysis, insecticidal activity and membrane binding.[10–12] The cyclotides can therefore be used either for their intrinsic bioactivity or be grafted with peptide sequences with a known pharmacological effect. This approach would utilise the stability of the cyclotides whilst at the same time improving the pharmacokinetics of the active peptide sequence.

The discovery of kalata B1 originates from traditional medicine in central Africa where *O. affinis* or "Kalata kalata" as the native name is, was used to facilitate childbirth. The compound itself was purified from the uterotonic fraction of the plant extract.[1,13] Despite the widespread interest in kalata B1 and other cyclotides, there has been very few *in vivo* studies performed and there is a lack of methods for the analysis of kalata B1 in biological matrices.[1,14] The development of selective and robust methods for the analysis of cyclotides in plasma and tissues is highly important for the continued investigation of the usefulness of these compounds in drug development. The currently published methods focus mainly on the analysis of cyclotides from plant extracts or are not sensitive enough to be optimal for peptide quantification in samples from *in vivo* experiments.[14,15] The principles of quantitative analysis of peptides in biological matrices has been reviewed by van den Broek et al in 2008 and highlights the specific considerations that must be taken when analysing peptides with LC-MS systems.[16]

The pharmacokinetics of peptides in general differs in certain important aspects from small molecular drugs, as reviewed by Diao and Meibohm in 2013.[17] Peptides usually have very short half-lives, which is caused by rapid degradation through hydrolysis. Another factor causing the short half-life of peptides is their effective clearance from the circulation by the kidneys. Peptide drugs are generally eliminated in the same way as endogenous peptides *i.e.* degraded to amino acids that are recycled for endogenous use. The volume of distribution of peptides has been reported to be very limited, usually to the volume of the extracellular space.[17] These properties provide challenges for the development of peptide drugs and need to be addressed for any peptide that is of interest for drug development. In this context, the advantages of cyclotides are obvious, as they overcome several of these problems. The pharmacokinetics of kalata B1 has not been reported in detail, apart from a calculation of the plasma half-life after intravenous dosing.[14] Describing the plasma pharmacokinetics and other parameters, such as tissue binding, provides a framework for further studies of the behaviour of kalata B1 and other cyclic peptides *in vivo*. Brain tissue binding is an important parameter to study as it will provide an early insight into the behaviour of these peptides in the central nervous system.

The aim of this paper was to develop a method for the quantitative analysis of kalata B1 in biological matrices that is robust and of high sensitivity, and which is amenable to small sample volumes and uses short runtimes. This would facilitate the analysis of high number of samples from in vivo studies aimed at assessing the plasma pharmacokinetics, as well as the brain pharmacokinetics of kalata B1.

2 | MATERIALS AND METHODS

2.1 | Materials

Kalata B1 was extracted and purified from the leaves of *Oldenlandia affinis* and the pure kalata B1 powder was dissolved in methanol. Acetonitrile (ACN), ethanol, methanol and formic acid (FA) were of gradient grade (Merck, Darmstadt, Germany). Deionized water was purified using a MilliQ Academic system (Millipore, Bedford, MA). Phosphate buffer (PBS) at pH 7.4 was prepared in house. Disodium phosphate (Na_2HPO_4), sodium dihydrogen phosphate (NaH_2PO_4 x H_2O), and sodium chloride for buffer preparation were all from Merck.

2.2 | Animals

Male Sprague Dawley rats were used (Taconic, Denmark). All animal experiments were performed in accordance with the guidelines from the Swedish National Board for Laboratory Animals, and were approved by the Animal Ethics Committee of Uppsala, Sweden (Ethical approval C188/14 and C16/12). The animals were housed in groups with *ad libitum* access to food and water and with a 12 h light-dark cycle, and were allowed to rest for 7 days before the study start. All surgery was performed one day prior to experiments and done under isoflurane anaesthesia (Baxter Medical AB, Kista, Sweden). Catheters were placed in the femoral vein and artery for dosing and sampling.

3 | INSTRUMENTATION

3.1 | LC system

Liquid chromatography separation was performed using an LC-10ADvp pump and a SIL-HTc autosampler (Shimadzu, Kyoto, Japan) which were connected to a HyPurity C18 column (50 ×4.6 mm, particle size 3 μm), protected by a guard-column of the same material (10 × 4.0 mm) (Thermo Scientific, MA). The mobile phases used for the chromatography were A: MilliQ Water containing 0.05% FA, and B: ACN:water (90:10 v/v) containing 0.05% FA. Flow rate was 0.8 mL/min at an operating pressure around 800 PSI, with 0.3 mL/min entering the MS system after splitting the flow. Separation was performed using the gradient 10% - 55% B over 6 minutes. The gradient was then reversed back to 10% B between 6 and 6.5 minutes.

3.2 | MS system

Electrospray ionisation, using positive ionisation mode, was performed on a Quattro Ultima triple quadrupole mass spectrometer (Waters, Milford, MA). Mass spectra were processed using the MassLynx software, version 4.1 (Waters).

Tuning was performed to optimize the detector parameters in order obtain the highest sensitivity possible, by direct infusion of kalata B1 dissolved in mobile phase at a flow rate of 0.2 mL/min using a Harvard 22 syringe pump (Harvard Apparatus Inc. Holliston, MA). The desolvation temperature was set to 450°C and the source temperature to 130°C. The desolvation gas (N_2) flow was maintained at 1000 L/h and the cone gas (N_2) flow was maintained at 40 L/h. The cone voltage was set to 140 V and the capillary voltage was 3.8 kV. The analysis was performed in the multiple reaction monitoring (MRM) mode monitoring the 1446.8 → 1446.8 m/z transition.

4 | STANDARD AND QC PREPARATION

Stock solutions of kalata B1 (1 mg/mL) were prepared in methanol and stored at −20°C. Standards were prepared in blank rat plasma and rat brain homogenate. Plasma standards were prepared at eleven concentrations in the range of 2 − 10,000 ng/mL and four QC levels (7.5, 75, 750, and 7500 ng/mL). Brain homogenate standards were also prepared at eleven concentrations in the range of 5 − 2000 ng/g brain with three QC levels (12, 120, and 1200 ng/g brain). Brain standards were prepared by adding four parts phosphate buffered saline (PBS) to one part brain and homogenized using a VCX ultrasonicator (Sonics & Materials, CT). Standards and QCs were aliquoted to polypropylene vials and stored at −80°C.

5 | SAMPLE PREPARATION

For both brain and plasma assays, samples were precipitated with three times their volume in acetonitrile, usual volumes being 50 μL sample and 150 μL acetonitrile. The samples were vortexed and then centrifuged for three minutes at 10000 rpm using a Scanspeed mini (Labogene, Lynge, Denmark). Then, 150 μL of the supernatant was transferred to new tubes and dried under N_2 at 40°C. Dry samples were reconstituted with 100 μL of mobile phase A, before being transferred to autosampler vials. Samples were kept at 5°C before 40 μL of the sample was injected onto the LC-MS system.

6 | METHOD VALIDATION

The method was validated according to the FDA guidance of validation of bioanalytical methods (revision 1).[18] All concentrations were determined by comparing the peak area of unknown samples and QCs to nominal concentrations of the calibration curve. A weighting factor, $1/y^2$, was used and the calibration curve was not forced through the origin. For both matrices the intra-day accuracy as well as precision were determined by analysing all standards and six replicates of each QC during one day. The LLOQ was determined by analysing five replicates of two LLOQ candidates during the same run as the standards and QCs. Precision was determined by the coefficient of variation (CV), expressing the standard deviation as a percentage of the mean concentration. The accuracy was described as the deviation of the experimentally determined concentration to the nominal concentration, again expressed as a percentage. Six blank samples of each matrix were also analysed to determine the specificity of the method. This was done by investigating the presence of interferences at the same retention time as that for kalata B1.

7 | APPLICATION OF THE METHOD

The method was applied to determine the plasma pharmacokinetics of kalata B1 in male Sprague Dawley rats (Taconic, Lille Skensved, Denmark), as well as assessing binding of kalata B1 to brain homogenate. The animal was given a 1 mg/kg dose of kalata B1 as a short, 10 min, infusion, and blood samples (200 μL per sample) were taken over four hours, with dense sampling around the maximal concentration to fully capture the pharmacokinetic profile, all samples were prepared as described above. Pharmacokinetic parameters, such as half-life and clearance, were then calculated using non-compartmental analysis of the data from the pharmacokinetic study.

The method was also applied to measure the binding of kalata B1 to brain homogenate with equilibrium dialysis (ED). In short, ED uses a semipermeable membrane to assess the binding of a compound to the homogenate, prepared by mixing one part rat brain with four parts of PBS. The homogenate was spiked with 1 μM of kalata B1. A Teflon 96-well plate model of the HTD96b ED-apparatus (HTDialysis, CT) was used for the experiment. Semipermeable membranes with a molecular weight cut-off of 12 − 14 kDa were used (HTDialysis, CT). Membranes were soaked in ethanol the day prior to the experiment, and left overnight in PBS. After assembling the apparatus, 125 μL brain homogenate was added on one side of the membrane and 125 μL buffer on the other side. The ED apparatus was equilibrated at 37°C for 24 h in an incubator with orbital shaking (MaxQ4450 Thermo Fisher Scientific, MA, set at 200 rpm. Samples of 80 μL were taken from both sides of the membrane after 6 or 24 h, and analysed as described above. Buffer samples were mixed with equal volumes of blank homogenate, and homogenate samples were mixed with equal volumes of blank buffer to avoid matrix effects influencing the results. The unbound fraction was calculated by dividing the concentration in the buffer by the concentration in the homogenate. The calculated unbound fraction was corrected for the dilution factor (D) using *Equation* 1 to compensate for the dilution of the brain homogenate.[19]

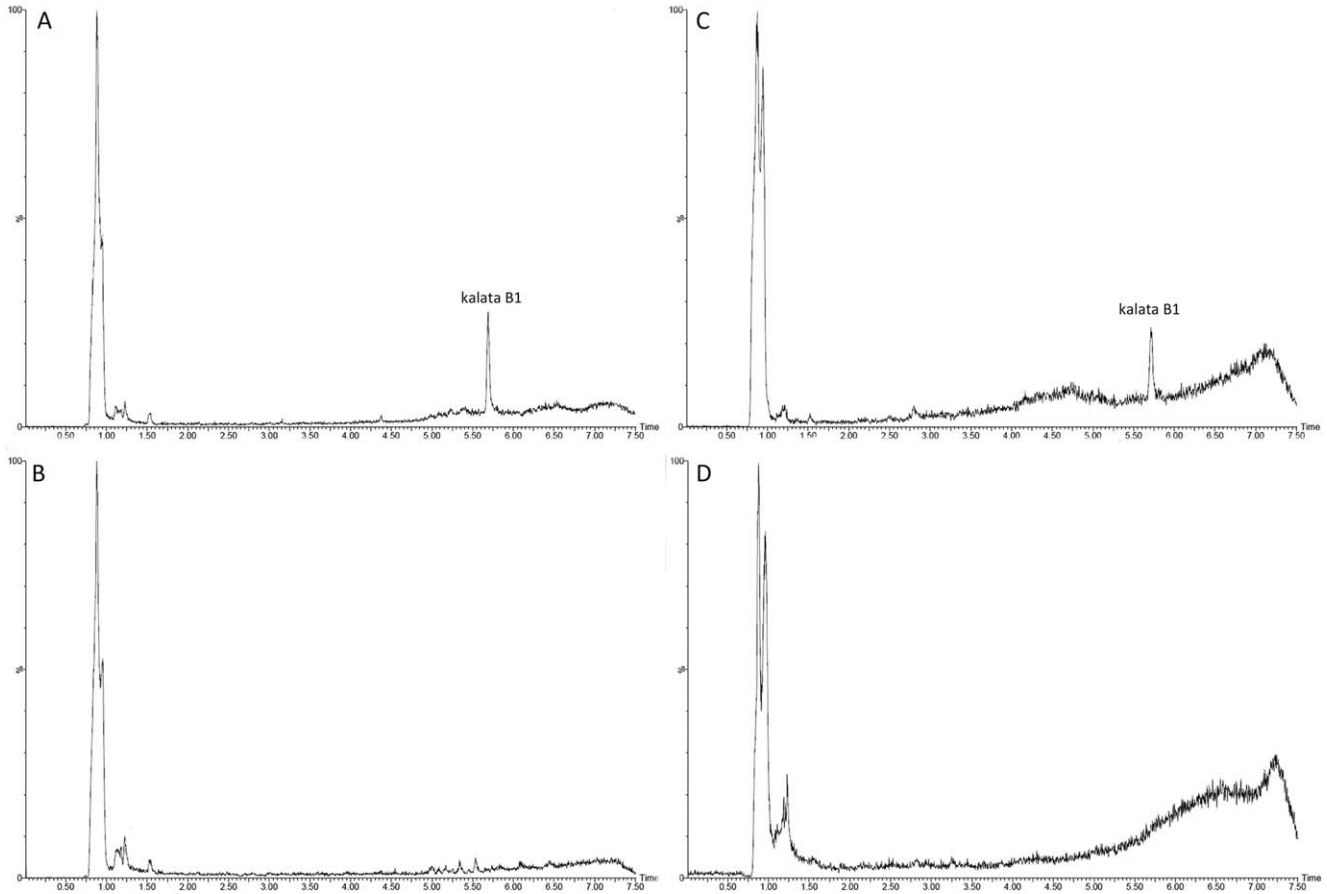

FIGURE 1 Specificity for kalata B1 in rat plasma and brain homogenate. A: 10 ng/mL kalata B1 in rat plasma. B: Blank rat plasma. C: 25 ng/g kalata B1 in brain homogenate. D: Blank rat brain homogenate. The double charged ion of kalata B1 is marked by kalata B1. The *y*-axis shows relative intensity (0 − 100 %), the X-axis shows retention time (0 − 7.5 min)

$$\text{Undiluted } f_u = \frac{1/D}{\left(\left(\frac{1}{f_u}\right) - 1\right) + 1/D} \quad (1)$$

The unbound fraction of kalata B1 in plasma was determined using the same principles as for brain homogenate.

Data was analysed using GraphPad Prism version 6.01 for Windows (GraphPad Software, San Diego, CA), and R Studio (R version 3.2.2, https://www.R-project.org).

8 | RESULTS AND DISCUSSION

8.1 | Analytical procedure

Gradient composition and slope was optimized to achieve optimal separation and peak shape, while keeping the runtimes low. The final ratio of the mobile phases was set to 45:55 (A:B), starting at 10:90 (A:B). The gradient was run over 6 min, and the kalata B1 peak was eluted after 5.7 min. The amount of formic acid in the mobile phases was optimized with regards to signal strength and retention time. The tested concentrations were 0.1, 0.05, and 0.01%. Optimal response as well as retention was obtained at 0.05%. Using trifluoroacetic acid as an additive instead of formic acid caused signal suppression, in line with earlier observations.[16]

The most intense ion observed during MS analyses in the current experimental setup was the doublecharged ion of kalata B1 (m/z 1446.8, $[M + 2H]^{2+}$) .Other ions were observed, e.g. $[M + 3H]^{3+}$ and $[M + 2H + K]^{3+}$, but neither of these had as high intensity as $[M + 2H]^{2+}$ and that ion was therefore selected for monitoring. Due to the high stability of kalata B1, fragmentation was not possible without compromising the signal strength and the MRM method was therefore set to monitor the parent ion through both quadrupoles.

It was noted that the method is very sensitive to contamination, with a peak often occurring with a retention time similar to kalata B1. After a literature search it was found that a peptide originating from human keratin has the same mass as the studied ion.[20] Thus, special measures to ensure the purity of the samples and minimize the contamination had to be taken, and therefore performing the sample preparation in a fume hood is recommended.

The method shows high specificity for kalata B1 (Figure 1) and the sensitivity was improved compared to the

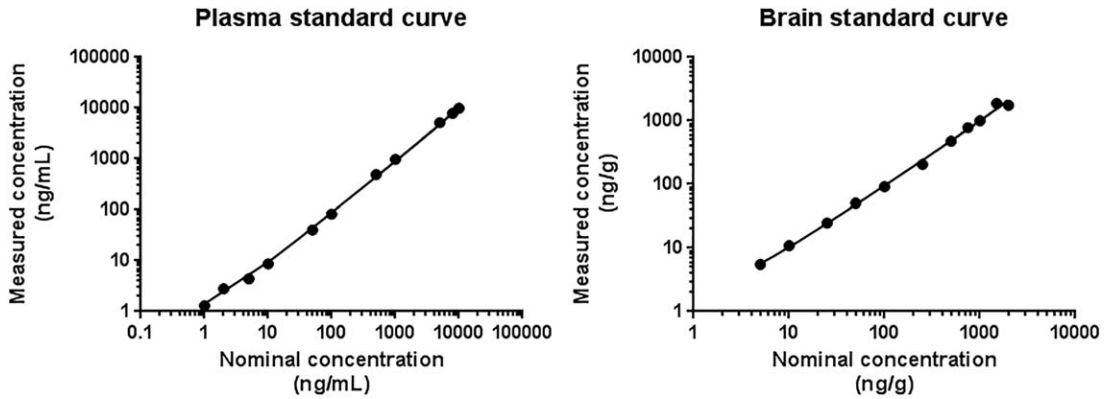

FIGURE 2 Standard curves of kalata B1 in plasma and brain homogenate. The *R*2 value for both curves was > 0.995

previously published method quantifying kalata B1 in plasma.[14] The LLOQ for plasma was determined by assessing two LLOQ levels, 2 ng/mL and 5 ng/mL. The 2 ng/mL samples had a CV of 11% and an accuracy of 105%. It was therefore acceptable as LLOQ, falling within the LLOQ definition of having a precision and accuracy equal to or below 20%. Brain homogenate LLOQ was assessed in the same way and was determined to be 5 ng/g, with a CV of 13% and an accuracy of 89%.

9 | VALIDATION

Method validation was performed in accordance with FDA guidelines. The method showed linearity in the entire range for both standard curves with r-squared values > 0.995 for both brain and plasma (Figure 2). The precision was assessed by analysing six replicates of QC samples at four different concentrations in plasma and three different concentrations in brain homogenate. The CV was found to be below 15% for all QC levels in both plasma and brain homogenate (Table 1). Accuracy was measured by dividing the experimentally determined QC concentrations by the nominal concentration of said QC. Again, four QC levels were used for plasma and three QC levels were used for brain. The accuracy was above 89% for all QC levels, *i.e.,* below the threshold value of 15% deviation. Inter-day precision and accuracy was also assessed, with the precision CV being below 15% for the lowest QC level and below 7% for the higher levels. The accuracy was above 88% for all QC levels.

10 | APPLICATION OF THE METHOD

The method was applied to quantify concentrations of kalata B1 in plasma to determine pharmacokinetics *in vivo* as well as *in vitro* brain samples. The low LOQs enabled quantification of kalata B1 in biological samples several hours after administration and after a low dose (1 mg/kg), giving more accurate estimates of the pharmacokinetic parameters. This

TABLE 1 Validation data for kalata B1 in plasma and brain homogenate

		Inter-day			Intra-day	
Compound/matrix	Nominal concentration (ng/ml)	Measured Concentration (ng/ml)	CV (%)	Accuracy (%)	Measured Concentrations (ng/ml)	CV (%)	Accuracy (%)
Kalata B1/plasma	7.5	6.7	6.4	89.8	7,2	15	96.3
	75	70	8.7	93.6	70	7.1	93.6
	750	670	7.0	89.3	667	6.9	88.9
Kalata B1/brain homogenate	7500	7725	4.7	103	7542	5.6	101
	12	11.3	11	94	10.9	13	91
	120	127	4.7	105	114	14	95
	1200	1187	7.4	99	1193	14	99

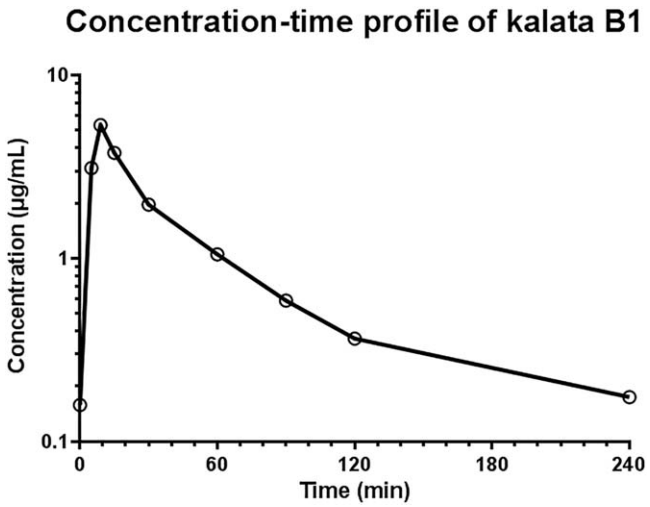

FIGURE 3 Example of a concentration time profile of kalata B1 in rat plasma. Concentration shown on a logarithmic axis in µg/mL. Open circles represent individual blood samples, connected with a solid line, $n = 1$. After a dose of 1 mg/kg the concentrations are well over the LLOQ of the developed method for all samples, showing that the method is adequately quantify the low concentrations needed to correctly describe the pharmacokinetics of kalata B1

enabled the administration of even lower doses (0.25 mg/kg), minimizing the risk for haemolysis and other possible toxicities associated with kalata B1.

The terminal half-life in plasma was determined to 113 minutes, and the volume of distribution to 241 mLmL/kg body weight. The calculated half-life differed from a previously reported *in vivo* half-life of kalata B1, as the half-life reported in this study was the terminal half-life from the elimination phase of the concentration-time profile, i.e calculated from the slope of the last few data points, since kalata B1 displays two- or possibly three-compartment kinetics. Samples taken at later time-points in this study might also contribute to the difference in half-life. Compared to other peptides, this is a relatively long half-life, and it is reasonable to assume that this is due to the high stability of the kalata B1 structure. This makes the molecule less susceptible to enzyme degradation, a major reason behind the usually low half-life of peptides. The volume of distribution was higher than the vascular volume in rats (40 mL/kg), indicating that kalata B1 is distributed also to peripheral tissue. The concentration-time profile of kalata B1 can be found in Figure 3.

The high sensitivity allowed for sampling also at later time points, making more accurate pharmacokinetic analysis possible as the whole concentration time curve could be captured.

The quantification of kalata B1 in brain homogenate was performed on samples after ED. Samples were taken at 6 and 24 h to determine that equilibrium was reached. It was found that equilibrium was reached after 24 h but not at 6 h, and those samples were further used to determine the unbound fraction.

The fraction of unbound drug in brain homogenate was found to be 0.13 ± 0.08, based on five replicates. This relatively high binding to brain tissue correlates well with findings that kalata B1 associates with cell membranes.[21] The level of plasma protein binding was assessed using the same method and the fraction unbound in plasma was found to be 0.25 ± 0.08, based on 5 samples.

Attempts were made to apply the method for samples collected in brain microdialysis experiments. These attempts have so far proved unsuccessful as the dialysate buffer appears to suppress the signal of kalata B1 to such an extent that the method was unable to quantify relevant concentrations or handle the small sample volumes obtained from microdialysis. Whether this can be solved is so far unclear as the reason for the suppressed signal is unknown.

11 | CONCLUSION

A new quantitative method with improved sensitivity for the analysis of kalata B1 is described for both plasma and brain homogenate. The methods were validated and show good precision and accuracy, as well as high specificity for the compound of interest, kalata B1. Care should be taken not to contaminate the samples with keratin from skin debris during processing. By providing a method for quantifying the concentration of kalata B1 in both plasma and brain homogenate we have established a base to determine the transport of kalata B1 and similar peptides across the blood-brain barrier. This method will aid research on the use of kalata B1 in drug development.

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