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Quantitative assay for bradykinin in rat plasma by liquid chromatography coupled to tandem mass spectrometry

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ABSTRACT

An assay to quantify bradykinin in rat plasma has been developed and validated, using liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS). Sar-D-Phe[8]-des-Arg[9]-bradykinin was used as internal standard. Aprotinin was added to rat plasma to inhibit the activity of proteinases. Recoveries for solid-phase extraction (SPE) on Strata X reversed phase were greater than 80%. Multiple reaction monitoring (MRM) on a triple quadrupole mass spectrometer equipped with an electrospray source (ESI), operating in the positive ion-mode, was used for detection. The assay was validated and stability was explored. Bradykinin (10–500 ng/mL) was quantified with accuracy values (% RE) below 10% and intra- and inter-day precisions (% RSD) below 12 and 16%, respectively, for all concentrations. The method was successfully applied to several plasma samples from low levels kallikrein rats (LKRs) compared with normal kallikrein rats (NKRs).

1. Introduction

Plasma bradykinin (BK), cleaved from high molecular weight kininogen by kallikrein, is a vasoactive peptide involved in cardiorenal physiology and inflammatory states. Profiling changes in the concentration of this peptide hormone is important for understanding the physio-pathology of hypertension. A reduction of kallikrein, the enzyme responsible for BK synthesis, has been described in various animal models of genetic hypertension [1,2]. Rats deficient in kininogen, a precursor of bradykinin, show salt-induced hypertension [3]. On the contrary, transgenic mice that overexpress human tissue kallikrein are chronically hypotensive [4,5]. In recent years, our laboratory has selectively developed a rat strain by breeding rats on the basis of low urinary kallikrein levels. We hypothesized that this rat strain has lower amounts of kinins than the control strain. In accordance with this hypothesis, this strain shows, as well as low levels of kallikrein, higher sensitivity in developing hypertension upon external stimuli such as dietary salt excess [1] or cadmium administration [6]. Moreover, this strain also displays an altered renal phenotype with polydipsia, polyuria and increased GFR [7]. For these reasons, we believed that the kallikrein–kinin system defect may have caused increased sensitivity to endogenous Angiotensin II in low kallikrein rats. This hypothesis was confirmed in a recent study [8] in which we observed that LKRs have a statistically significant elevation in Angiotensin II levels compared to controls, thereby providing even further evidence that the low kallikrein rats strain has low levels of kinins. Radio-Immuno-Assay (RIA) is the traditional method employed for the quantification of BK in plasma [9–11]. Although immunological methods can be highly sensitive, there is a risk of cross-reactivity, given that the antibodies used cannot discriminate between structurally related peptides. Accurate quantification is therefore limited, and data should be interpreted cautiously [12]. Reported BK serum or plasma concentrations measured by immunoassays vary largely between the nanomolar and the picomolar range [13]. Other assays, using LC–MS, have been applied to quantify bradykinin in rat muscle tissue dialysate [14], or bradykinin's stable metabolite, BK 1-5, in human blood [15]. Van den Broek et al. in a recent work describing a LC–MS/MS method for the quantification of bradykinin and other peptides in human patients reports plasma concentrations in the order of ng/mL [16]. Nowadays, mass spectrometry is the method of choice for sensitive and selective detection of peptides. This technique can discriminate co-eluting peptides with different masses, while performing tandem mass spectrometry even allows compound-specific masses of fragments to be detected and used for quantification [12]. In this work, an analytical method for the quantification of bradykinin in rat plasma is presented, in order to measure the peptide hormone levels in two rat strains, namely,

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low (LKRs) and normal (NKRs) level kallikrein rats. To date, the determination of BK levels has been hampered by its artificially high concentration in drawn blood, due to the fact that activation of the kallikrein–kinin system is induced by vascular trauma and negatively charged surfaces (e.g. glass) of blood collection containers [11–13]. Yi et al. report that immediate exposure of blood samples to protease inhibitors may enable the intrinsic plasma BK dosage [17]. Therefore, aprotinin, which inhibits serine proteases with a particularly high affinity for plasma kallikrein, was added to rat blood samples during blood collection. Moreover, to minimize the loss of bradykinin, experiments were performed using polypropylene vials. The proteolysis-resistant BK analogue Sar-D-Phe^8-des-Arg^9-bradykinin was used as internal standard (IS) for the method's development.

2. Experimental

2.1. Chemicals and reagents

Bradykinin acetate, trifluoroacetic acid (Reagent Plus 99%), methanol (HPLC grade), acetonitrile (gradient grade) and formic acid (puriss. p.a.) were purchased from Sigma–Aldrich (Milan, Italy). Sar-D-Phe^8-des-Arg^9-bradykinin (≥95%) was obtained from Phoenix Europe GmbH (Karlsruhe, Germany). Aprotinin (Protease inhibitor) was supplied by MP Biochemicals (Verona, Italy). Bovine plasma was obtained from Innovative Research (Novi, MI, USA). Deionized and distilled water was purified through a MilliQ water system (Millipore, Bedford, MA, USA). Strata X (100 mg/mL) columns for solid phase extraction (SPE) were purchased from Phenomenex (Torrance, CA, USA) and mounted on a VacElut vacuum manifold (Supelco at Sigma–Aldrich, Milan, Italy).

2.2. Animals

Experiments were performed in a rat strain with low levels of kallikrein. This strain originates from an outbred Wistar colony (Morini, Reggio Emilia, Italy) selected exclusively for its low urinary kallikrein levels [18], and currently shows a significant difference in urinary kallikrein excretion compared to normal kallikrein Wistar rats (Morini), which were used in this work as a control. All animals (23 LKRs and 23 NKRs), weighing 280–300 g, were housed at constant room temperature (24 ± 1 °C) and humidity (60 ± 5%) with a 12-h light–dark cycle, and had free access to standard rat chow (Mucedola, Milan, Italy) and tap water. Before blood collection, all animals were housed in individual metabolic cages to collect 24-h urine by determining kallikrein urinary activity. During the collection periods, rats had free access to water but were deprived of food. Blood samples were collected from anesthetized animals, and each rat was humanely killed after blood collection. All procedures complied with the standard stated in the *Guide for the Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources, National Academy of Sciences, Bethesda, MD, USA) and were approved by the local Animal Care and Use Committee.

2.3. Urinary kallikrein activity

Urinary volume was determined gravimetrically. Urinary kallikrein activity was determined by amidolytic assay [19], which was modified by the addition of soybean trypsin inhibitor (SBTI; Sigma Chemical Company, Milan, Italy) in the incubation buffer.

2.4. LC–MS/MS conditions

LC–MS/MS analyses were performed using a ProStar TM 300 HPLC system (Varian, Palo Alto, CA, USA) coupled with a Varian 310-MS triple quadrupole mass spectrometer. Samples were injected into a 5 μl loop. The analytes were separated using reversed-phase liquid chromatography on a Varian Polaris C18-A, 5 μm, 100 mm × 2.0 mm column (Palo Alto, CA, USA) fitted with a 3 μm Polaris C18-A security guard cartridge (10 mm × 2.0 mm ID) (Varian Inc., Palo Alto, CA, USA). An elution gradient with two solvents was used: (A) water with 0.1% formic acid and (B) acetonitrile. A linear gradient was performed as follows: 3 min at 5% solvent B; in 0.5 min solvent B was increased from 5 to 20%, and remained constant at 20% B for 3.50 min. Then in 1 min solvent B was decreased from 20 to 5% and it remained constant for 4 min. The total run time was 12 min for each injection. All chromatographic solvents were degassed online with a vacuum degasser from Varian. The flow-rate was set at 0.3 mL/min. Detection was carried out using a triple quadrupole system, a 310 LC–MS/MS spectrometer, from Varian, fitted with an electrospray interface (ESI) operating in positive mode. The ESI source conditions were: capillary voltage, 5000 V; drying gas temperature, 200 °C; nebulizer gas pressure, 50 psi (both nebulizer and drying gas were high-purity nitrogen); electron multiplier voltage, 1500 V.

For operation in MS/MS mode, the collision gas used was argon with a pressure of 2 mTorr in the collision cell. For the optimization of detection conditions, direct infusion of standard solutions (250 ng/mL) was made using a T connection with mobile phase at a flow rate of 400 μL/min. Individual parameters for bradykinin and internal standard are listed in Table 1.

2.5. Preparation of standards, internal standard and quality control samples

The stock solution (100 μg/mL) of BK reference standard was used to prepare 10 μg/mL working solutions (one for the calibration standards and the other for quality control (QC) sample preparation). Stock and working solutions were prepared in methanol/water/formic acid (50:50:0.1, v/v/v) and stored at −80 °C prior to use. Standard samples were prepared in bovine plasma, and QC samples were prepared in rat plasma. Standard concentrations of 10, 25, 50, 100, 250 and 500 ng/mL were prepared by serial dilution of a freshly prepared 1000 ng/mL solution in bovine plasma.

QC samples at concentrations of 10, 25, 200 and 400 ng/mL were prepared by appropriate dilution of a 10 μg/mL BK working solution in rat plasma and stored in a −80 °C freezer.

The proteolysis-resistant BK analogue, Sar-D-Phe^8-des-Arg^9-bradykinin, was used as internal standard. The internal standard working solution was prepared in methanol/water/formic acid (50:50:0.1, v/v/v) at a concentration of 5 μg/mL and stored at −80 °C prior to use.

2.6. Sample preparation

Blood samples were collected into Vacutainer tubes containing EDTA, and rocked several times immediately after collection to prevent coagulation. Blood was then transferred into polypropylene tubes containing aprotinin (0.6 TIU/mL of blood) to inhibit the activity of proteinases. After centrifugation at 1610 × *g* for 15 min at 4 °C, plasma was collected and stored at −80 °C until analysis. 1 mL of plasma added to 20 μl of IS working solution was acidified with 1 mL of milliQ water containing 1% trifluoroacetic acid (buffer A), and was then centrifuged at 13200 × *g* for 20 min at 4 °C. The supernatant was loaded onto a Strata X cartridge which had been previously conditioned with 1 mL of 60% acetonitrile in 1% trifluoroacetic acid (buffer B) and 3 mL of buffer A. After washing of the cartridge with 3 mL of buffer A, the analyte was eluted with 2 mL of buffer B into a polypropylene tube. After evaporation under nitrogen stream, the sample was reconstituted in 1 mL of milliQ water.

Table 1
MS/MS settings for bradykinin and internal standard.

Peptide	RT (min)	Capillary (V)	MRM transitions (*m*/*z*)	CE (eV)
Bradykinin	5	55	531.5 → 531.5	8.5
		45	531.5 → 70.0	34
Sar-D-Phe^8-des-Arg^9-bradykinin	5.8	45	488.6 → 713.8	20

RT: retention time; MRM: multiple reaction monitoring; CE: collision energy.

2.7. Method validation

Validation was based on the FDA guidelines for Bioanalytical Method Validation [20].

Calibration standards were prepared in bovine plasma. Six calibration standards were measured in concentrations ranging from 10 to 500 ng/mL. All standards were analysed in three separate runs, and target peptide IS ratios were calculated for each concentration level. Sar-D-Phe^8-des-Arg^9-bradykinin was used as IS. Calibration curves were derived from the peak area ratios (bradykinin/internal standard) using $1/x^2$ weighted linear least-squares regression of the area ratio versus the concentration of the corresponding standard. The precision and the accuracy of the method were evaluated at QC concentrations over the linear dynamic range (10, 25, 200, 400 ng/mL). Precision was expressed as the percent relative standard deviation (% RSD), where the sample standard deviation (s) was calculated for six replicates for each level for the within-day (intraday) precision and over 3 days for the between-day (interday) precision. Intra- and inter-day accuracy was expressed as relative error percentage (% RE).

The method was also assessed to determine matrix effect on ion suppression or enhancement using six different samples of bovine plasma and rat plasma fortified to contain 10 ng/mL of bradykinin. Peak areas of endogenous and exogenous compounds co-eluting with the analyte or internal standard should be less than 20% of the peak area of the LLOQ standard and less than 5% of the response of the IS. The selectivity of the method was evaluated by analysing six different bovine plasmas with and without IS.

The extraction recovery was evaluated analysing in triplicate three different concentrations (10, 25, and 200 ng/ml) by comparing the peak areas of bovine plasma samples spiked before extraction with bovine plasma samples spiked after extraction. To determine total recovery, peak areas of the bovine plasma samples spiked before and after extraction were also compared to the peak areas of reference samples in elution solvent.

Stability experiments were performed in rat plasma: to evaluate freeze/thaw stability, two QC samples at concentrations of 10 and 200 ng/mL were subjected to three freeze–thaw cycles including defrosting (5 min) at room temperature and freezing at −80 °C for at least 12 h. Long-term stability was evaluated after the QC samples were stored in −80 °C freezer for 90 days. The samples were then analysed using freshly made calibration standards. Furthermore, BK stability in the stock and working solutions was examined: in the working solutions, BK was assessed after 24 h at room temperature and after one month of storage at −20 °C, whereas in the stock solutions, BK was assessed after two months of storage at −80 °C. Relative deviations were calculated by comparing MS response ratios to freshly prepared samples at identical concentrations. All stability tests were performed in triplicate. The analytes were considered stable when 85–115% of the initial concentration was found.

3. Results and discussion

In this work, an analytical method for the quantification of bradykinin in rat plasma is presented, and has been applied to measure the peptide hormone levels in two rat strains, namely, low (LKRs) and normal (NKRs) level urinary kallikrein rats. The LKR strain showed a decrease in urinary kallikrein (7.5 ± 3.2 nKat/24 h) of around 2-fold compared to rats with normal kallikrein excretion (18.3 ± 4.1 nKat/24 h).

3.1. Method development

A method entailing application of liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS) after solid phase extraction with Strata X cartridges was developed and validated for BK analysis. The solid phase extraction for sample preparation was important to obtain satisfactory accuracy and precision for the plasma BK assay. We found that the Strata X SPE cartridge provided the best retention for bradykinin compared to other SPE options. Different gradients of acetonitrile and 0.1% formic acid were assayed at a constant flow rate of 0.3 mL/min. Acetonitrile was chosen as the organic solvent because it provided a higher sensitivity and lower background noise than methanol. To separate interfering compounds from bradykinin we have optimized a gradient HPLC elution using a Polaris C18A.

Mass spectra were acquired using direct infusion of each standard in 0.1% HCOOH, applying ESI ionisation sources in both negative and positive mode. These preliminary experiments showed effective results only in positive ESI mode.

Fig. 1A shows the ESI mass spectrum of bradykinin at a concentration of 100 ng/mL. Bradykinin predominantly yielded the protonated molecular ion at m/z 531.5, corresponding to charge states of 2+. Fig. 1B shows the ESI mass spectrum of the BK analogue Sar-D-Phe^8-des-Arg^9-bradykinin, resulting in a similar profile which predominantly contained the 2+ molecular ion. The BK molecular ion corresponding to the 2+ charge state (m/z 531.5) was selected for BK quantification, whereas another abundant product ion at m/z 70 was used for the additional confirmation of identity. Since bradykinin is an endogenous peptide hormone, it was present in rat plasma. Therefore, another animal plasma was evaluated as a surrogate matrix to calibrate the assay, given that it does not contain BK. The employ of surrogate biomatrix for the quantification of endogenous compounds is described in the literature [16]. For this study, we chosen the bovine plasma as the surrogate matrix because it has been demonstrated the cleanest matrix among those tested. In addition, the bradykinin is susceptible to non-specific binding to container surfaces (e.g. glass), especially when the compound was present at low concentrations [11–13,17]. Therefore to minimize loss of bradykinin, which results in poor responses and non-linear correlations, experiments were performed using polypropylene vials.

3.2. Method validation

The calibration curves showed linearity in the range of 10–500 ng/mL. Average correlation coefficient (r^2), slope and intercept obtained after three curves were 0.995 ± 0.007, 0.092 ± 0.02, −0.1098 ± 0.05, respectively. The lower limit of quantification (LLOQ), defined as the lowest concentration that could be measured with a precision better than 20% RSD and accuracy within ±20%, was 10 ng/mL. Accuracy (% RE) and precision (% RSD) were between −2.5 and 9.6% and between 8.8 and 15.8%, respectively (Table 2).

Table 2
Intra- and inter-day accuracy (% RE) and precision (% RSD).

QC i.d.	QC nominal concentration (ng/mL)	Intra-day results ($n = 6$)		Inter-day results ($n = 6$)	
		% RE	% RSD	% RE	% RSD
LLOQ	10	4.7	10.3	9.6	15.8
Low	25	−1.6	11.9	5.7	13.2
Medium	200	−2.5	9.9	−1.5	9.7
High	400	6.8	8.8	8.0	10.6

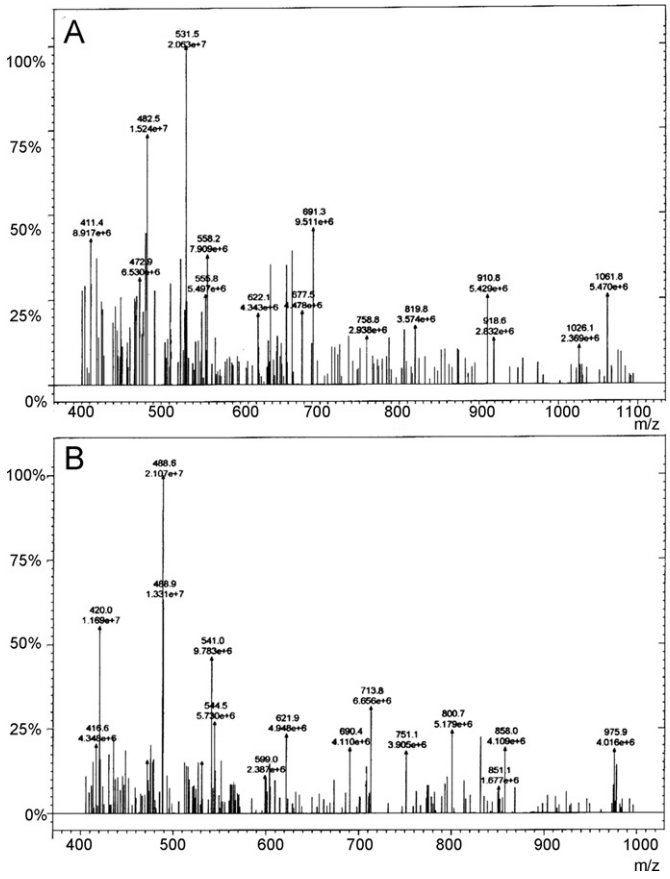

Fig. 1. ESI mass spectrum of bradykinin (A) and Sar-D-Phe[8]-des-Arg[9]-bradykinin (B).

No significant carryover (≤20% of the LLOQ) was observed. Matrix effect was evaluated by preparing spiked samples of 10 ng/mL BK in six different samples of bovine and rat plasma. The mean accuracies (% RE) in bovine plasma were between −10.5 and +7.5%, as shown in Table 3. The studies in rat plasma resulted in accuracies with between −13.5 and +9.5% RE.

Table 3
Matrix effect.

Plasma matrix	Spiked conc. (ng/mL)	Mean determined conc. (ng/mL, $n = 3$)	Mean % RE ($n = 3$)
Bovine	10	8.95	−10.5
	10	10.28	2.8
	10	9.65	−3.5
	10	10.75	7.5
	10	9.22	−7.8
	10	9.47	−5.3
	10	10.82	8.2
Rat	10	9.05	9.5
	10	10.50	5.0
	10	9.74	−2.6
	10	8.65	−13.5
	10	9.12	−8.8

Selectivity was evaluated in six different lots of rat plasma, with analysis performed with and without the internal standard. Selectivity was satisfactory as no interfering substances at the appropriate migration times were observed when six plasma samples were analysed.

The mean SPE extraction recovery for bradykinin was greater than 80% for all concentrations tested. Representative chromatograms of a standard solution and spiked rat plasma at a concentration of 100 ng/mL are shown in Figs. 2 and 3.

Freeze–thaw and long-term stability were evaluated in rat plasma. Bradykinin appears to be stable after three freeze–thaw cycles (mean % RE was between −7.3 and −1.3%) and also after storage at −80 °C for 90 days (mean % RE was between −4.0 and −2.8%). Results for stock and working solution stability were derived by comparing mean peak area ratios (BK/IS) of the stability solutions to mean peak area ratios of freshly prepared solutions. The BK stock solution was found to be stable (acceptance criteria of ±10%) for two months in methanol/water/formic acid (50:50:0.1, v/v/v) when stored at −80 °C. Bradykinin was stable in the working solution after one month storage at −20 °C (mean % RE was −0.1%) whereas it was rapidly degraded after 24 h at room temperature.

3.3. Rat plasma analysis

The described method has been applied successfully for BK determination in plasma samples from LKR compared with NKR. Fig. 4 shows BK concentrations in rat plasma obtained from 46

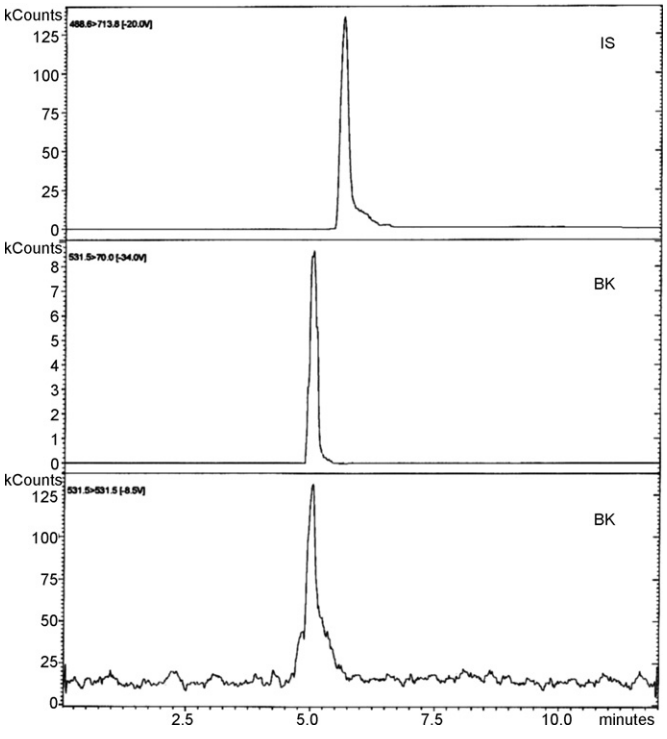

Fig. 2. Chromatogram of BK and IS solution at a concentration of 100 ng/mL.

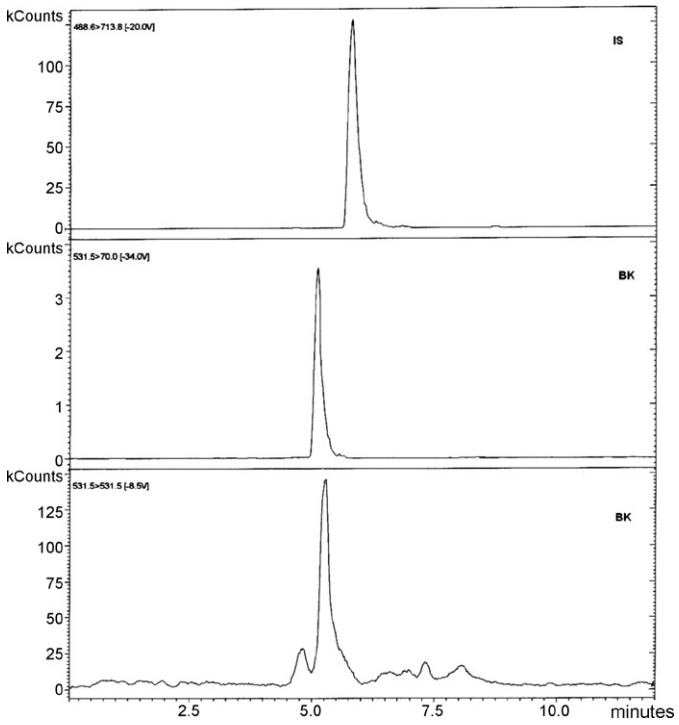

Fig. 3. Chromatogram of spiked rat plasma at a concentration of 100 ng/mL.

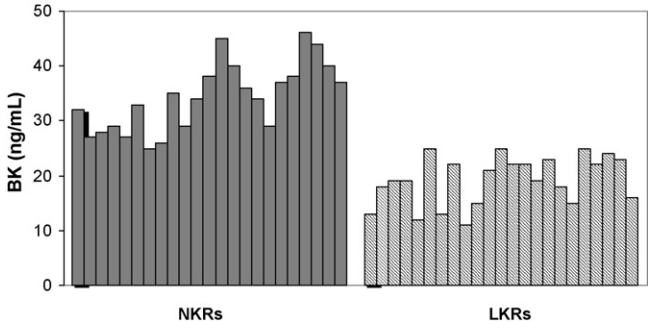

Fig. 4. BK concentrations (ng/mL) in rat plasma obtained from 23 LKRs and 23 NKRs.

samples (23 LKRs and 23 NKRs). In contrast to NKRs, BK concentrations in LKRs are lower; the average plasma BK concentration was 34.3 ± 1.29 ng/mL in NKRs versus 19.2 ± 0.92 ng/mL in LKRs. The average 2-fold decrease in BK observed in the LKR population versus normal subjects indicated that BK concentrations are correlated with a decrease in urinary kallikrein.

The detected concentrations of bradykinin were higher than those reported in the literature with RIA analysis (pg/mL) [9–11] whereas they are similar to BK levels (low ng/mL) detected by LC–MS/MS [16]. On the other hand, various factors can affect the *ex vivo* formation of bradykinin (e.g. vascular trauma and negatively charged surface of blood containers induces the activation of the kallikrein–kinin system) and these are still unclear [17].

4. Conclusions

We have described a fully validated LC–MS/MS method for the determination of bradykinin in rat plasma using MS/MS detection and a solid phase extraction for sample preparation prior to LC–MS/MS measurement. Bradykinin was quantified using a chemically synthesized BK standard and the BK analogue Sar-D-Phe^8-des-Arg^9-bradykinin as internal standard in bovine plasma as the surrogate calibration matrix. The assay is capable of measuring bradykinin over the range of 10–500 ng/mL. This method allowed unambiguous detection of rat BK compared to the RIA assay, where cross-reactivities prevent a definitive measurement of this peptide. The method has been successfully applied to real rat plasma.

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