HIGH PERFORMANCE LIQUID CHROMATOGRAPHY OF BRADYKININ AND ITS
APPLICATION TO THE DEGRADATION STUDY OF BRADYKININ IN PLASMAYayoi HIRAGA, Kazuhide SHIRONO, Sachiko OH-ISHI*,
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High performance liquid chromatographic separation and
fluorometric determination of bradykinin and two analogs with
alkaline ninhydrin reagent, are described. Complete separation
of bradykinin, des-Arg⁹-bradykinin and des-Phe⁸-Arg⁹-bradykinin
was achieved in a single step by reversed phase high performance
liquid chromatography. The fluorescence development is due to
the reaction of guanidino groups of arginine residues with the
ninhydrin reagent. The procedure is highly sensitive and
specific, and was applied to the study of degradation of
bradykinin in plasma.

For a long time, pharmacologists have needed a sensitive, selective and
specific method for microassay of bradykinin (BK) and its metabolites. Since
trace amounts of BK exert remarkable biological activities, a specific bioassay
has been widely used for BK¹⁾. In this method, metabolites, such as des-Arg⁹-
BK, do not interfere with the assay because they do not cause contraction of rat
uterus or guinea pig ileum. Some metabolites do have biological activities,
e.g. causing contraction of arterial strips, but the application of such
activities to the assay of metabolites has not been studied²⁾. Radioimmunoassay³⁾
or enzyme immunoassay⁴⁾ has been used for the measurement of BK, but for assay
of metabolites of BK by these methods, it is necessary to raise antisera to each
metabolite separately.

High performance liquid chromatography (HPLC) has advantages over these
methods in that it allows simultaneous determination and identification of BK
and its metabolites. Recently, Marceau et al.⁵⁾ reported the analysis of BK and
its metabolites by HPLC using a UV monitor. However, other components may
interfere with the UV detection of BK in biological samples.

We recently reported the HPLC determination of guanidino compounds using
alkaline ninhydrin reagent⁷⁾. This reagent reacts with compounds containing a
guanidino group, such as creatinine and arginine, and the reaction is not
influenced by other amino acids or peptides that do not contain a guanidine
residue. We examined whether this method could be used for microdetection and
determination of BK and its metabolites, which contain an arginine residue.

This report describes the application of this method to the determination of BK and its metabolites in biological fluids and to the study on degradation of BK by plasma.

MATERIALS AND METHODS

Reagent

BK was purchased from Sigma Chemical (St. Louis, MO, USA). Des-Arg⁹-BK and des-Phe⁸-Arg⁹-BK were from Protein Research Foundation (Osaka, Japan). Captopril (CP) was the gift from Sankyo Pharmaceutical (Tokyo, Japan). All other reagents were of analytical reagent grade and they were purchased from Wako Pure Chemical (Osaka, Japan).

Chromatographic system

Figure 1 shows the chromatographic system. A Zorbax-ODS column (150 mm x 4.6 mm I.D.; DuPont Instruments, Wilmington Del., USA) thermostated at 50°C in a water bath was used for separating components. The mobile phase was a mixture of a 100 mM aqueous trisodium citrate dihydrate solution : acetonitrile : methanol (72 : 10 : 18). The pH of the mobile phase was adjusted to 3.0 with a 60 % perchloric acid solution. The flow rate of this mobile phase was kept at 1.5 ml/min using an LC-3A pump (Shimadzu, Kyoto, Japan).

To the effluent were delivered first 2 N NaOH, and then a 0.6 % aqueous ninhydrin solution both at a constant flow rate of 0.5 ml/min using a multiple channel peristaltic pump (SWP-101, Sanuki Industry, Tokyo, Japan). The mixture of the effluent with alkaline ninhydrin reagent was then passed through a PTFE tubing reaction coil (10 m x 0.5 mm I.D.) in a water bath at 50°C, and its fluorescence intensity was measured with an Shimadzu RF-530 spectrofluoromonitor using excitation and emission wavelengths of 395 nm and 500 nm, respectively. The excitation lamp was a xenon discharge lamp (75 W; Wacom R & D Corp., Kumagaya, Japan).

Fig. 1 Flow diagram of HPLC system

* Zorbax ODS (150mm x 4.6 mm I.D.); ** 10 m x 0.5 mm I.D.;

*** Shimadzu RF-530

Degradation of BK and des-Arg⁹-BK by plasma

Human blood was collected by venipuncture from healthy men in syringes containing 10 units of heparin/ml of blood. The plasma was either used immediately or stored at -20°C until use.

A mixture of 42 nmol of BK or des-Arg⁹-BK in 0.5 ml of 50 mM Tris-HCl buffer (pH 7.4) containing 0.1 M NaCl and 0.1 ml of plasma was incubated at 37 °C for 1-3 h. Then 0.1 ml of a 30 % trichloroacetic acid solution was added, the mixture was centrifuged for 5 min at 1000g, and 25 µl of the supernatant solution was applied to the HPLC column described above.

RESULTS and DISCUSSION

Chromatographic separation and quantitative measurement of BK and its analogs

BK, des-Arg⁹-BK and des-Phe⁸-Arg⁹-BK could be separated by reversed phase HPLC, and were detected by their fluorescence on reaction with alkaline ninhydrin reagent, as shown in Fig. 2A. The retention times of des-Phe⁸-Arg⁹-BK, BK and des-Arg⁹-BK were 6, 25, and 35 min, respectively. Figure 2B shows the elution pattern of normal human plasma on the same column. The peak in the chromatogram was probably due to low molecular weight guanidino compounds, such as creatinine and arginine, and was well separated from those of BK and its analogs.

Fig. 2 Chromatogram of a mixture containing 1 nmol of each peptide (A) and that of normal human plasma (B)

Linear relations were obtained between the peak heights and amounts of the peptides in ranges of 0.05–5 nmol BK, 0.1–5 nmol des-Arg⁹-BK, and 0.05–5 nmol desPhe⁸-Arg⁹-BK. The lower limits for detection of these peptides were 25, 50 and 25 pmol, respectively. The coefficients of variation (n=7) in measurements on samples of 1 nmol and 2.5 nmol of standard BK were 2.1 % and 2.5 %, respectively, and those of 1 nmol and 2.5 nmol of des-Arg⁹-BK were 1.0 % and 2.2 %. The recoveries of 1 nmol of BK and des-Arg⁹-BK (n=6) added to human plasma were 90.5 % (C.V. = 2.1 %) and 86.2 % (C.V. = 3.2 %), respectively.

Degradation of BK and des-Arg⁹-BK by normal plasma

Figures 3A, B and C show the chromatograms obtained after incubation of BK with normal plasma at 37 °C for 0 (A), 1 (B) and 3 (C) h. Peak 3 had the retention time of BK, and peaks 4 and 2 had those of des-Arg⁹-BK and des-Phe⁸-Arg⁹-BK, respectively. The material in peak 1 may be a shorter peptide. The results of Figure 3 show that BK is cleaved by plasma enzymes to des-Arg⁹-BK and des-Phe⁸-Arg⁹-BK and a shorter chain peptide.

Fig. 3 Chromatographic profiles obtained after incubation of BK with human plasma. Peak 1: unidentified; peak 2: des-Phe⁸-Arg⁹-BK; peak 3: BK; peak 4: desArg⁹-BK. Samples of 42 nmol of BK in 0.5 ml of 0.05 M Tris-HCl buffer containing 0.1 M NaCl and 0.1 ml of human plasma were incubated at 37 °C and then deproteinized with 30 % trichloroacetic acid. Time of incubation: A, 0 h; B, 1 h; C, 3 h

Kininase I and kininase II are the main enzymes in human blood that inactivate BK¹⁾, and they also inactivate BK when injected into the blood of animal *in vivo*. Kininase I cleaves carboxy terminal arginine, and kininase II, the angiotensin converting enzyme (CE) which is abundant in lungs, cleaves Gly-Phe bonds, and so splits Phe-Arg from BK. Using an amino acid analyser, Cicilini et al.⁷⁾ observed the formation of des-Arg⁹-BK, des-Phe⁸-Arg⁹-BK, Phe-Arg, Phe, and Arg on degradation of BK by plasma. However, Marceau et al.⁵⁾ reported that des-Phe⁸-Arg⁹-BK was not produced from BK by CE because CP, which inhibits CE, did not affect its formation. Thus they suggested that it was formed by successive attacks on BK by kininase I; i.e., two-step degradation from the C-terminus.

Figure 4A shows a chromatogram of the mixture of des-Arg⁹-BK (peak 4) and plasma incubated at 37 °C for 1 h. Figure 4B shows a chromatogram of the same incubation mixture, but with a standard des-Phe⁸-Arg⁹-BK (peak 2) added just before the application of the mixture to the column. Des-Phe⁸-Arg⁹-BK was not observed in Fig. 4A. This fact indicates that, contrary to the conclusion of Marceau et al.⁵⁾,

Fig. 4 Chromatographic profile obtained after incubation of des-Arg⁹-BK with human plasma at 37 °C for 2 h. (A) and that with addition of 0.5 nmol of des-Phe⁸-Arg⁹-BK just before chromatography (B)
Peak 1: unidentified material; peak 2: des-Phe⁸-Arg⁹-BK; peak 4: des-Arg⁹-BK
For conditions see "MATERIALS AND METHODS"

des-Phe⁸-Arg⁹-BK cannot be formed via des-Arg⁹-BK. Similar results on degradation products were obtained using four other specimens of normal human plasma. On the other hand, a shorter chain peptide (peak 1) appeared at the same retention time with peak 1 in Fig. 3. Preliminary experiment suggested that the peptide in peak 1 is a pentapeptide Arg-Pro-Pro-Gly-Phe which is formed by enzymic cleavage of des-Arg⁹-BK. The detail of this experiment will be published in the near future⁸⁾.

Figure 5 shows the chromatograms obtained after incubation of BK with human plasma in the presence of various concentrations of CP. The formation of des-Phe⁸-Arg⁹-BK (peak 2) and the unidentified material (peak 1) were only slightly inhibited by 3.3 μ M CP (B), as shown by comparison with

Fig. 5 Chromatograms of BK incubated with human plasma at 37 °C for 1 h in the presence of various concentrations of CP. Concentration of CP: A, 0 μ M; B, 3.3 μ M; C, 33 μ M; D, 330 μ M. Peak 1: unidentified; peak 2: des-Phe⁸-Arg⁹-BK; peak 3: BK; peak 4: des-Arg⁹-BK

the control without CP (A). The formation of des-Phe⁸-Arg⁹-BK was apparently inhibited by 33 μ M CP but the formation of des-Arg⁹-BK from BK was little affected (C), indicating that only CE activity was inhibited at this concentration of CP. The degradation of BK to give des-Arg⁹-BK and des-Phe⁸-Arg⁹-BK was affected by 330 μ M of CP, indicating both kininase I and CE activities were affected at this concentration of CP.

The above results suggest that 462 nM CP used by Marceau et al.⁵⁾ was too low to inhibition of CE. Moreover, they did not tried the incubation of des-Arg⁹-BK with plasma. In the present study, we showed that des-Arg⁹-BK was further degraded to a shorter peptide (Fig. 4), and that this degradation was inhibited by CP suggesting that the enzyme which degradetes des-Arg⁹-BK resembles CE. Thus the present study clarified that des-Phe⁸-Arg⁹-BK is generated directly from BK by the action of CE, not from des-Arg⁹-BK by the action of kininase I.

Patterns of BK degradation by normal and uremic plasma

Figure 6 shows the chromatograms of BK incubated with normal plasma (A) and plasma of a patient with chronic glomerulonephritis before (B) and after (C) hemodialysis. Samples of plasma (0.1 ml) were incubated with 42 nmol of BK at 37°C for 1 h. Uremic plasma before dialysis was more active in conversion of BK to des-Arg⁹-BK than normal plasma and its activity was reduced by hemodialysis.

Fig. 6 Chromatograms obtained after incubation of BK at 37° C for 1 h with normal human plasma (A) and plasma from a uremic patient* before (B) and after (C) hemodialysis. Peak identities are given in Fig. 5

*Clinically stable chronic uremic patient (male, aged 37 years) receiving hemodialysis three times a week.

normal plasma, and its activity was reduced by hemodialysis. This finding suggests that the kininase I activity of the uremic plasma is higher than that of normal plasma. No significant change in plasma CE activity in this patient was observed⁶⁾. Similar results were obtained with plasma samples from seven other patients with chronic glomerulonephritis. These findings suggest that the present method should be useful for examination of enzyme activity involved in BK metabolism in cases of various diseases.

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