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Study of bradykinin metabolism by rat lung tissue membranes and rat kidney brush border membranes by HPLC with inductively coupled plasma – mass spectrometry and orthogonal acceleration time-of-flight mass spectrometry

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Abstract: The coupling of the techniques, high-performance liquid chromatography (HPLC), orthogonal acceleration time-of-flight mass spectrometry (OATOF-MS) and inductively coupled plasma mass spectrometry (ICP-MS) provides a very powerful method for identifying and quantifying the products of bradykinin metabolism. In this study, we were able to identify the major metabolites of bradykinin degradation reported in the literature. In addition, a new bradykinin metabolite corresponding to bradykinin 5,9 fragment (BK-(5,9)-fragment) was identified as a product of neutral endopeptidase (NEP) activity. This finding establishes that NEP cleaves bradykinin simultaneously at the positions 4–5 and 7–8. We also demonstrate the equivalent participation of NEP and angiotensin-converting enzyme (ACE) within the rat lung tissue membranes (RLTM) in bradykinin degradation, suggesting its suitability as a model for the assay of dual ACE/NEP inhibitors. On the contrary, in rat kidney brush border membranes (KBBM), ACE is not significantly involved in bradykinin metabolism, with NEP being the major enzyme. Copyright © 2005 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: bradykinin metabolism; NEP; ACE; ICP-MS; mass spectrometry

INTRODUCTION

Bradykinin (BK) is a small peptide that acts mainly as a hormone by activating specific receptors that confer protection against the development of hypertension. The role of BK and its metabolism has been well studied and reviewed by several authors [1–6]. The efficacy of BK is influenced by the activities of various kininases belonging to the metallopeptidases genre. Several metabolic studies have shown that the metallopeptidases that participate in BK metabolism vary by location (e.g. plasma, tissues), the physiological conditions, as well as the species. Angiotensin-converting enzyme (ACE) and neutral endopeptidase (NEP) have been reported as two of the more important metallopeptidases involved in BK metabolism. The ACE/NEP (single and dual) inhibition principles and its clinical perspectives have been reviewed in the literature [7–9].

In a previous study [10], we developed a new analytical method that combines high-performance liquid chromatography (HPLC) on-line with orthogonal acceleration time-of-flight mass spectrometry (OATOF-MS) and inductively coupled plasma mass spectrometry (ICP-MS) to investigate in human and rat plasma the metabolism of labelled BK with $^{79/81}$Br on Phe in position 5 and also $^{15}N_2$ on Arg in position 1. Using this approach, we obtained more information than that provided by the traditional methods. We have previously demonstrated [11] that rat plasma is not a suitable *in vivo* model for the evaluation of ACE/NEP inhibition in human plasma. In addition, it is known that NEP, which is a membrane-bound enzyme, is not present in plasma, thus its activity or inhibition cannot be studied. In order to determine the contribution of NEP enzyme towards the metabolism of BK, we undertook experiments with samples enriched with cell membranes. In the present work, we have applied the HPLC-OATOF/ICP-MS methodology to investigate the bromobradykinin (BrBK) degradation in rat lung tissue membranes (RLTM) and rat kidney brush border membranes (KBBM). In both tissues we confirm the presence of NEP.

Abbreviations: ACE, angiotensin-converting enzyme; APP, aminopeptidase P; BK, bradykinin; BrBK, bromobradykinin; CPN, carboxypeptidase N; DPPIV, dipeptidyl peptidase IV; EBSS, Earle's balanced salt solution; HPLC, high performance liquid chromatography; ICP-MS, inductively coupled plasma mass spectrometry; KBBM, rat kidney brush border membranes; NEP, neutral endopeptidase; OATOF-MS, orthogonal acceleration time-of-flight mass spectrometry; POP, prolyl oligopeptidase; RLTM, rat lung tissue membranes; TFA, trifluoroacetic acid.

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MATERIALS AND METHODS

Chemicals

BK and the synthetic BrBK (H-Arg($^{15}N_2$)-Pro-Pro-Gly-Phe(4-Br$^{79/81}$)-Ser-Pro-Phe-Arg-OH) were purchased from Neosystem (Strasbourg, France). Earle's balanced salt solution (EBSS), HEPES, captopril and hippuryl-L-histidyl-L-leucine hydrate (hip-his-leu) were purchased from Sigma-Aldrich (Irvine, UK). Trifluoroacetic acid (TFA) was obtained from Acros (New Jersey, USA) and formic acid from BDH (Poole, UK). The HPLC grade acetonitrile used in the chromatography was purchased from Fisher Scientific (Loughborough, UK) and the ultrapure water was produced from an Elga water purification system (Elga, High Wycombe, UK). Pure recombinant rabbit NEP and pure rabbit ACE were provided internally at GSK.

Preparation of RLTM and KBBM

Lung and kidney tissues were removed from pentobarbital anaesthetised rats and placed in ice-cold saline. Centrifugation steps were carried out at 4 °C. KBBM were prepared as described by Edwards *et al.* [12]. Briefly, kidneys were bisected and inner and outer medulla tissues were discarded. Cortices were homogenised using a polytron in 2 mM HEPES pH 7.4, 50 mM mannitol, 35 ml per two kidneys. 1 M $MgCl_2$ was added to the homogenate to give a final concentration of 1 mM followed by stirring on ice for 20 min. Homogenate was then centrifuged for 10 min at 2000 g. Supernatant was centrifuged for 15 min at 35 000 g. The resulting pellet was resuspended in 5 mM HEPES pH 7.4, 300 mM mannitol and homogenised using a Teflon homogeniser.

This was centrifuged for 15 min at 35 000 g followed by resuspension of the fluffy white portion of the pellet. This step was repeated followed by resuspension in 50 mM Tris pH 7.5.

RLTM were produced as described previously by Nambi *et al.* [13], except that protease inhibitors and EDTA were deliberately omitted. Ten milliliters of buffer containing 20 mM Tris pH 7.5, 0.25 M sucrose was used to homogenise each gram of lung tissue using a motor-driven Teflon mortar and glass pestle homogeniser. Homogenate was centrifuged at 1000 g for 10 min. Pellet was discarded and supernatant was filtered through cheesecloth followed by centrifugation at 40 000 g for 30 min. This pellet enriched in cell membranes was suspended in 50 mM Tris pH 7.5.

Incubation Procedure

The protocol used was a modified version of that described by Dendorfer *et al.* [14] Briefly, the incubations were performed at 37 °C in a buffer solution supplemented (9 : 1 ratio) with EBSS. The buffer solution was composed of 50-mM HEPES, 150-mM NaCl and 1-μM Zn^{2+}. Either 5 μl of NEP (600 nM), ACE (100 nM), RLTM or KBBM was added to 1.155 ml of incubation buffer containing 10 μM of BrBK, without or with different concentrations of NEP/ACE inhibitors. Aliquots (110 μl) from the incubations were taken at appropriate time points, supplemented with TFA (2.2 μl) and centrifuged at 4 °C for 30 min at 2000 g. The samples were maintained at 4 °C and analysed as soon as practicable on the day of incubation.

HPLC-OATOF/ICP-MS Analysis

An improvement in the separation of the BrBK fragments generated by different enzymes was achieved on a Waters Symmetry column (C8, 3.5 μm) 50 × 2.1 mm (Waters, Milford, Massachusetts, USA). The sample injection volume was 5 μl. The flow rate of the LC system was 0.4 ml/min using a non-linear reversed phase gradient as depicted in Table 1. The mobile phase consisted of 0.1% formic acid in water (solvent A) and 0.1% formic acid in 90% acetonitrile/water (solvent B). The outlet flow of the LC pump was split equally between the OATOF-MS (LCT, Micromass, Manchester) and the ICP-MS (Platform Life, GV Instruments, Wythenshawe, UK) instruments. The identities of BrBK and its degradation products were elucidated by mass spectrometry and the quantification was performed by ICP-MS.

Quantification Measurements of BrBK and its Metabolites

Masslynx™ 3.4 software was used for data acquisition and processing for both the ICP-MS and OATOF-MS instruments. Quantification measurements were achieved by ICP-MS, which is an element-specific detector with almost uniform response independent of the structure of the metabolite [15]. In order to use ICP-MS, a synthetic form of BK was brominated in position 4 of the phenylalanine; we refer to this material as BrBK. Thus, the quantification of BrBK and its metabolites was based on the response of the detector to bromine. For all quantification studies, a calibration curve under identical conditions was produced with BrBK as a standard. The ICP-MS response was linear within the range of concentrations used throughout our experiments. The ICP-MS limit of detection of bromine under our conditions was 250 nM (100 pg on column). BrBK degradation kinetics were fitted with the monoexponential function: $BrBK = BrBK_0 e^{-kt}$, where k is the degradation rate and t the time. BrBK and $BrBK_0$ are the concentrations of BrBK at time t and time t_0 respectively.

Table 1 Non-linear reverse phase HPLC gradient used for the analysis of BrBK metabolites

Time (min)	% Solvent B
0	4
0.5	4
14	6.5
15	7.5
16	10.5
17	11.5
20	11.5
24	20
26	26
27	35
28	4
30	4

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RESULTS AND DISCUSSION

BrBK Degradation by ACE and NEP Pure Enzymes

According to the literature [1–6], six main metallopeptidases have been reported as being particularly important in the metabolism of BK (Figure 1). ACE cleaves at the 7–8 and 5–6 positions to produce the BrBK-(1–7)-fragment and BrBK-(1–5)-fragment respectively. NEP cleaves BK at the position 7–8 to also give the BrBK-(1–7)-fragment, which is then the subject of a secondary NEP cleavage at the position 4–5 to generate the BrBK-(1–4)-fragment and BrBK-(5–7)-fragment [16]. Aminopeptidase P (APP) cleaves at the position 1–2 to produce the BrBK-(2–9)-fragment. carboxypeptidase N (CPN) cleaves at the position 8–9 to produce the BrBK-(1–8)-fragment. Prolyl oligopeptidase (POP) is specific for cleaving the carboxylic side of proline bonds, and thus is able to cleave BK at the positions 7–8 and 3–4 to generate BrBK-(1–7)-fragment, BrBK-(4–9)-fragment and BrBK-(4–7)-fragment [2,17]. A similar but more specific activity has been reported for dipeptidyl peptidase IV (DPPIV), which cleaves the position 3–4 on des-Arg^1-BK [18], i.e. after BK has been cleaved by the APP enzyme.

In our previous studies [11] performed with rat and human plasma, BrBK-(1–5)-fragment and BrBK-(1–7)-fragment were chromatographically unresolved. In that instance, because of the absence of NEP in plasma, both fragments observed represented ACE activity, and the unresolved peaks did not represent a problem for our studies. By incubating pure enzyme preparations of NEP and ACE with BrBK, we have improved our chromatographic conditions. Figure 2a depicts the chromatogram for BrBK before the addition of either of the enzymes. Figures 2b and c depict the chromatograms for the resulting metabolites of BrBK degradation after 30 min incubation with NEP and ACE respectively. As can be observed in these figures, the BrBK-(5–7)-fragment, BrBK-(1–7)-fragment and BrBK-(1–5)-fragment are chromatographically well resolved, thus allowing us to determine and distinguish clearly NEP and ACE activities.

In our experiment with pure NEP enzyme and BrBK, we were able to identify a new metabolite, the BrBK-(5–9)-fragment (Figure 2b). This suggests that NEP has two primary sites of cleavage on BrBK, one at the 7–8 position (generating the BrBK-(1–7)-fragment fragment) and the other at the 4–5 position (generating the new BrBK-(5–9)-fragment). Following the primary NEP activity, secondary cleavage by NEP then occurs on both of the generated fragments (BrBK-(1–7)-fragment and BrBK-(5–9)-fragment) to produce the BrBK-(5–7)-fragment (Figure 2b). This finding was only possible thanks to the improved HPLC conditions, which are depicted in Table 1. Although we have already demonstrated [10] that there is no difference induced by the use of labelled BK (BrBK), the same experiment was performed with normal (non-labelled) BK and the BK-(5–9)-fragment was also identified. Figure 3 shows the isotopic pattern for the non-brominated (a) and brominated (b) BK-(5–9)-fragment. Considering this result, Figure 1 should be modified in all subsequent representations, such that the arrow showing NEP cleaving BK at the position 4–5 must be a solid line indicating a primary site of cleavage.

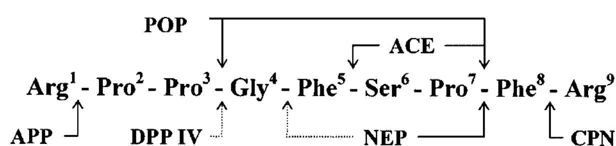

Figure 1 Enzymes implicated in the metabolism of BK and the sites of cleavage described in the literature. Solid lines are primary, independent cleavage sites. Dotted lines are secondary cleavage points dependent of previous hydrolysis of another bond.

Metabolism of BrBK by RLTM and KBBM

The metabolism of BrBK in the presence of RLTM or KBBM was monitored at different time points over a period of 180 min. Figure 4a shows an example of the chromatograms obtained in the presence of RLTM after 90 min of incubation. In addition to BrBK-(1–5)-fragment, BrBK-(1–7)-fragment, BrBK-(5–7)-fragment and BrBK-(5–9)-fragment obtained by ACE and NEP activities, BrBK-(1–8)-fragment and BrBK-(2–9)-fragment resulting from the CPN and APP activities respectively were identified. According to the literature [18], the fragment BrBK-(2–9)-fragment is further cleaved by DPPIV and POP to yield the BrBK-(4–9)-fragment. The formation of BrBK-(4–7)-fragment, BrBK-(4–5)-fragment and BrBK-(5)-fragment identified in this study could be explained by the combined action of APP, ACE/NEP, DPPIV and POP enzymes. Figure 4b shows an example of the chromatogram obtained in the presence of KBBM after 60 min of incubation where similar fragments to those found with RLTM (except BrBK-(4–5)-fragment, BrBK-(1–5)-fragment and BrBK-(1–8)-fragment) were characterised.

In terms of the metabolites produced during the BrBK degradation, major differences were observed between RLTM and KBBM. The activity of DPPIV or POP denoted by the formation of BrBK-(4–9)-fragment, BrBK-(4–7)-fragment and BrBK-(4–5)-fragment is very low in KBBM but very important in RLTM. In KBBM, the BrBK-(4–5)-fragment appears very late during the time course, after 120 min of incubation (data not shown). The BrBK-(1–5)-fragment representing ACE activity was not detected at all when incubating BrBK with KBBM, whereas this fragment is one of the major metabolites when using RLTM. We know that there is some low ACE activity in KBBM based on

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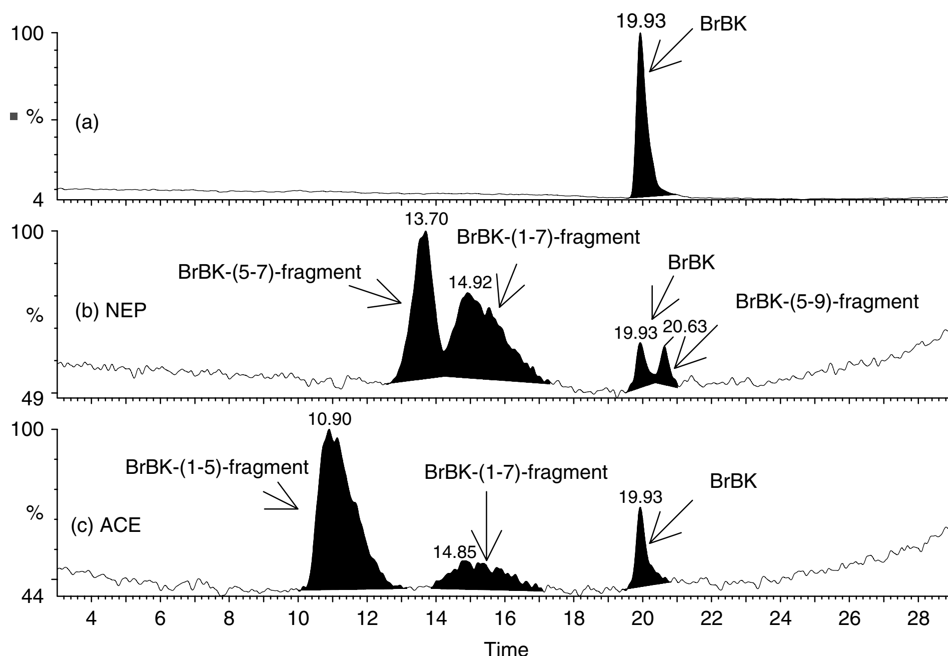

Figure 2 ICP-MS chromatograms for 10 μM of BrBK (a) and after 30 min incubation with NEP (b) and ACE (c).

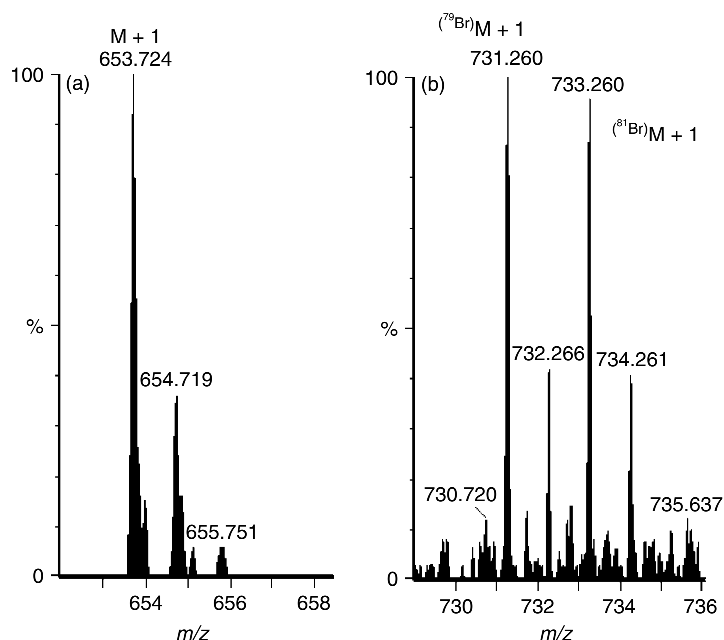

Figure 3 Isotopic pattern of the BK-(5–9)-fragment obtained from BK (a) and from BrBK (b) after its incubation with pure recombinant NEP enzyme.

experiments done with the specific ACE-substrate [19] hip-his-leu (data not shown), but under our HPLC-OATOF/ICP-MS conditions, if there is some production of BrBK-(1–5)-fragment, this is very low and below the limit of detection of our system. The BrBK-(1–8)-fragment from CPN was only detected when using the RLTM, which suggests the absence or low activity of CPN in KBBM. The pathway for the BrPhe (BrBK-(5)-fragment) production, detected in both tissues, is not yet fully established, but could be explained by the combined action of APP, ACE/NEP, DPPIV and POP enzymes.

ACE/NEP Inhibition in RLTM and KBBM

NEP and ACE activities in RLTM and KBBM were confirmed by using ecadotril and captopril as specific inhibitors of NEP and ACE enzymes respectively [20,21].

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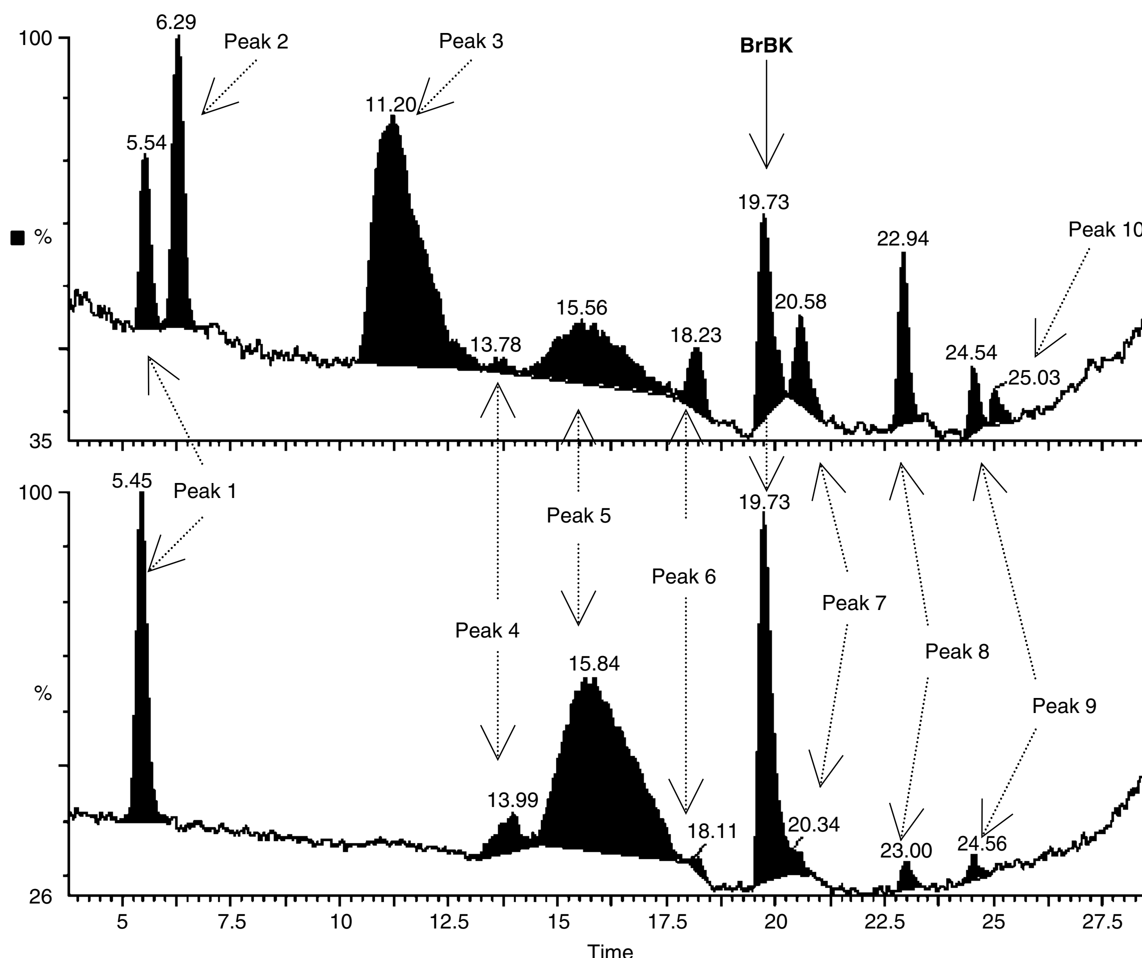

Figure 4 ICP-MS chromatograms for 10 μM of BrBK after 90 min incubation with RLTM (a) and after 60 min incubation with KBBM.
Key: Peak 1 = BrBK-(5)-fragment; Peak 2 = BrBK-(4–5)-fragment; Peak 3 = BrBK-(1–5)-fragment; Peak 4 = BrBK-(5–7)-fragment; Peak 5 = BrBK-(1–7)-fragment; Peak 6 = BrBK-(4–7)-fragment; Peak 7 = BrBK-(5–9)-fragment; Peak 8 = BrBK-(4–9)-fragment; Peak 9 = BrBK-(2–9)-fragment; Peak 10 = BrBk-(1–8)-fragment.

Omapatrilat was used as a dual ACE/NEP inhibitor [22]. In order to ensure the inhibition of the enzymes, different concentrations of each inhibitor were tested (data not shown). The concentrations presented in this paper were chosen because they resulted in total inhibition of ACE and NEP, i.e. abolishing the production of BrBK-(1–5)-fragment by ACE and that of BrBK-(5–9)-fragment and BrBK-(5–7)-fragment for NEP inhibition. The metabolism of BrBK in the absence or in the presence of the inhibitors was studied in RLTM and KBBM at different time points over a period of 180 min. Figure 5 summarises the half-life values of BrBK in the absence (control) and in the presence of ACE/NEP inhibitors for RLTM and KBBM.

RLTM

For the RLTM, similar values for BrBK half-life were obtained when using captopril (10 μM) or ecadotril (25 μM), 117 and 119 min respectively (see Figure 5a), threefold higher than that obtained for the control (40 min). This result suggests that in RLTM both ACE and NEP present a similar contribution towards BrBK degradation under our experimental conditions. When both enzymes are inhibited by omapatrilat (1 μM), the BrBK degradation is much slower with a half-life value (277 min) 6.9-fold higher than that obtained for the control and more than twofold higher than that obtained with each of the single enzyme inhibitors. Over the time course of our experiments in the presence of inhibitors, the generation of the other fragments due to APP (BrBK-(2–9)-fragment), CPN (BrBK-(1–8)-fragment) and DPPIV or POP (BrBK-(4–9)-fragment) was observed as for the control. The only difference was that these fragments were more stable (data not shown), suggesting the participation of ACE and/or NEP in their degradation. Under the ACE/NEP inhibition effect by omapatrilat (1 μM), only 30% of the initial BrBK was metabolised after 180 min of incubation. This observation confirms that ACE and NEP are the most important enzymes involved in BrBK degradation in the RLTM.

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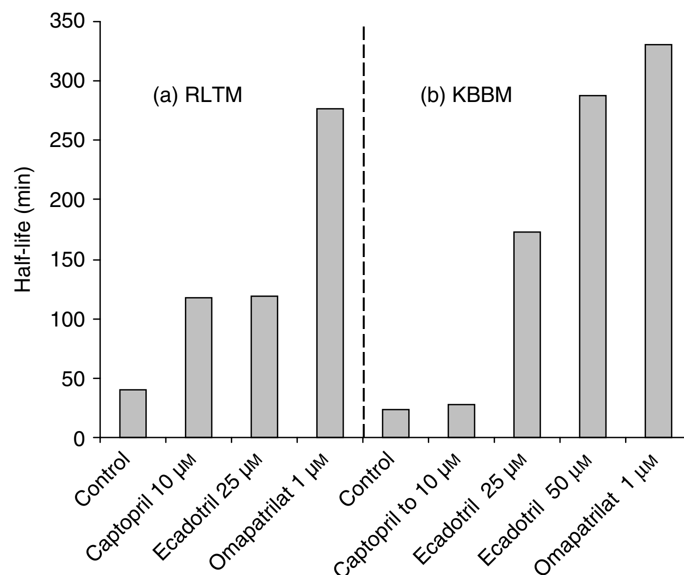

Figure 5 Half-life of BrBK after its incubation with (a) RLTM and (b) KBBM in the absence and presence of inhibitors.

KBBM

For the KBBM, no significant difference was observed for the half-life values between the control and the samples with captopril (10 μM), 23 and 28 min respectively (see Figure 5b). This result and the absence of the BrBK-(1–5)-fragment fragment during the study confirms the low participation of ACE in the BrBK degradation by our KBBM samples. The half-life value in the presence of the NEP inhibitor (ecadotril) was much higher. Initially, the same concentration was used (25 μM) as for the studies with the RLTM and the half-life value was 173 min (see Figure 5b), 7.5-fold higher than that for the control. Under these conditions (25 μM ecadotril), the production of BrBK-(1–7)-fragment and BrBK-(5–7)-fragment was not fully abolished. Therefore, the concentration of ecadotril was increased to 50 μM, and this completely inhibited NEP and gave a half-life value of 289 min (see Figure 5b), 12.6-fold higher than the control. This value is very similar to 330 min obtained with omapatrilat (see Figure 5b), which is 14.3-fold higher than that for the control. Over the course of our experiments in the presence of inhibitors, the generation of the other fragments due to APP (BrBK-(2–9)-fragment) and DPPIV or POP (BrBK-(4–9)-fragment) was as observed for the control. Once again, the only difference was that the fragments were more stable (data not shown), suggesting the participation of NEP in their degradation. Under the ACE/NEP inhibition effect by omapatrilat (1 μM), only 20% of the initial BrBK was metabolised after 180 min of incubation. Because the ACE activity is very low in KBBM, this last observation confirms that NEP is the most important enzyme involved in BrBK degradation in the KBBM.

CONCLUSIONS

After improving the chromatographic conditions, we were able to identify and characterise all the metabolites of the BrBK degradation. For the first time, we are reporting the BrBK-(5–9)-fragment as the result of NEP activity. Our results provide evidence that NEP cleaves BK at the 4–5 and 7–8 positions, primarily and simultaneously, rather than sequentially. The BrBK-(5–9)-fragment was also identified in RLTM and KBBM, both known to contain NEP. We have demonstrated that in terms of BrBK degradation, the contribution of ACE and NEP in the RLTM is quite similar; therefore, RLTM could be used as a model for the assay of dual ACE/NEP inhibitors. Because of the very rich content of NEP in the KBBM, this would make an excellent source for the study of NEP inhibitors.

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