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Investigation of Bradykinin Metabolism in Human and Rat Plasma in the Presence of the Dual ACE/NEP Inhibitors GW660511X and Omapatrilat

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Abstract: Several studies have suggested that the accumulation of bradykinin, or that of one its metabolites BK1–8, is involved in the occurrence of side effects such as AE associated with the use of various ACEi. In this work a novel approach combining HPLC-UV on-line with oaTOF-MS and ICPMS was applied to investigate in human and rat plasma the metabolism of labelled BK ($^{79/81}$Br-Phe^5) BrBK in the presence of two new dual ACE/NEP inhibitors (GW660511X and omapatrilat) currently under clinical trial.

In human plasma the BrBK half-life values in the absence or in the presence of GW660511X (3.8 μM) or omapatrilat (32 nM) were 38.7 ± 2.4, 51.2 ± 4.7 and 114.7 ± 9.3 min, respectively and BrBK was degraded into BrBK1–8, BrBK1–7, BrBK1–5 and Br-Phe [5]. In the presence of inhibitors, however, the levels of these resultant metabolites were different. Unlike GW660511X, omapatrilat abolished the production of BrBK1–5 and BrBK1–7, suggesting a better ACE inhibition effect over GW660511X as no NEP activity was found. In addition the production of BrBK1–8 was enhanced in the presence of these inhibitors with a greater accumulation being observed with omapatrilat. The production of Br-Phe^5 was reduced with GW660511X while no significant change was observed with omapatrilat after 4 h of incubation. In rat plasma the BrBK half-life values in the absence or in the presence of GW660511X (530 nM) or omapatrilat (50 nM) were 9.31 ± 1.7, 22.06 ± 3.1 and 25.3 ± 1.7 min, respectively and BrBK was degraded into BrBK1–8, BrBK1–7, BrBK1–5 and Br-Phe^5 plus BrBK2–9, BrBK4–8 and BrBK2–8 metabolites not found in human plasma. GW660511X and omapatrilat reduced the production of BrBK1–5 and BrBK1–7 with more effect being observed with omapatrilat. GW660511X and omapatrilat increased the production of both BrBK1–8 and Br-Phe^5 but not that of BrBK4–8 and BrBK2–8.

This study shows that the potency of GW660511X in comparison with omapatrilat is more than 100-fold lower in human, but less than 10-fold lower in rat plasma, suggesting that rat may not be a suitable

Abbreviations: ACE, angiotensin converting enzyme; ACEi, angiotensin converting enzyme inhibitor; AE, angiodema; APP, aminopeptidase P; BK, bradykinin; BrBK, bromobradykinin; CP, carboxypeptidase; DPAP, dipeptydylaminopeptidase; EBSS, Earle's balanced salt solution; HEPES, (N-[2-hydroxyethyl]piperazine-N′-[2-ethanesulfonic acid]); HPLC, high pressure liquid chromatography; ICPMS, inductively coupled plasma mass spectrometry; NEP, neutral endopeptidase; oaTOF/MS, orthogonal acceleration time-of-flight mass spectrometry; Phe, phenylalanine.

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in vivo model for the evaluation of ACE/NEP inhibition in relation to effects in humans.

Keywords: bradykinin; bradykinin metabolism; dual ACE NEP inhibitors; angiodema; ICPMS; mass spectrometry

INTRODUCTION

Bradykinin belongs to the kinin family of bioactive peptides released from their precursors known as kininogens [1] and exerts its action by activating the B2 receptors that are ubiquitous in mammalian tissues [2]. A lot of interest has been paid to BK because it plays an important role in the physiopathology of asthma, diabetes and is also involved in blood pressure regulation [2,3]. The metabolism of BK has been extensively studied [4] and it may be linked to the nature of the biological milieu, the physiological conditions, as well as to the species. Typically, several enzymes are involved in the metabolism of BK in plasma [5–9]. Figure 1 shows the structure of BK and its breakdown by ACE, CP N, NEP, and APP.

ACEi belong to the class of cardiovascular drugs that are well-established therapies for the treatment of essential hypertension and congestive heart failure [10]. However, their use in the clinic is limited because they have been associated with the occurrence of side effects such as AE [11,12]. The mechanism of AE remains unresolved, however, several studies performed in rats or in humans suggested that accumulation of BK and that of its metabolite BK1–8 in plasma may participate in the occurrence of AE [12–14]. Because it is important to have active but also safe compounds, a new class of cardiovascular drugs that simultaneously inhibit both ACE and NEP has been developed (for a review see ref [15]). Among this class of new compounds, GW660511X (Z13752A) (Figure 2A) has shown interesting properties at its early stage of evaluation [16,17] as well as omapatrilat (Figure 2B) [18,19]. Although this class of compounds appear to represent a more effective antihypertensive agent than ACEi alone [17,19], it is important to have information on their toxicity profile.

In our previous study, we developed a new analytical method that combines HPLC-UV online with oaTOF-MS and ICPMS to investigate in human and rat plasma the metabolism of labelled BK containing a bromine ($^{79/81}$Br-Phe^{5}) on Phe in position 5 (Figure 3) [20]. Using this approach we found that the selectivity and sensitivity were improved over traditional methods such as HPLC and UV detection and that the quantification of BrBK and its metabolites as well as their characterization were obtained using only one brominated compound. Thus, in the present work we applied this approach to investigate in human and rat plasma the metabolism of BK in the absence or presence of two new dual ACE/NEP

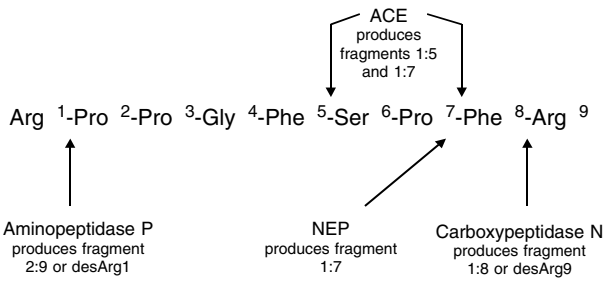

Figure 1 Major sites of cleavage and the enzymes implicated in the metabolism of bradykinin (BK).

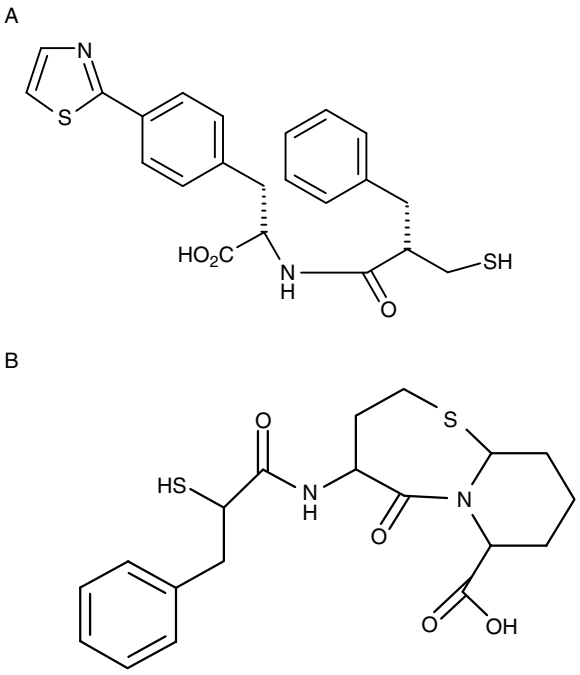

Figure 2 Structures of the dual ACE/NEP inhibitors used in this study (A) GW660511X (Z13752A), (B) omapatrilat.

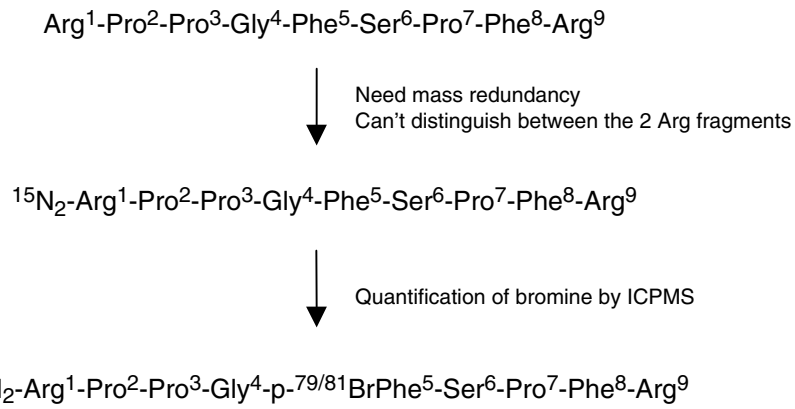

Figure 3 Structure of bromobradykinin (BrBk) used in this study.

inhibitors (GW660511X and omapatrilat) currently under clinical trial. This will enable us to determine the profile of the various BK metabolites, which could be indicative of their involvement in side effects of ACE/NEP inhibitors.

MATERIAL AND METHODS

Chemicals

The synthetic BrBK (H-$^{15}N_2$Arg-Pro-Pro-Gly-*p*-$^{79/81}$BrPhe-Ser-Pro-Phe-Arg-OH) was purchased from Neosystem (Strasbourg, France). Earle's balanced salt solution (EBSS), HEPES, apstatin, captopril and APP 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 produced from an Elga water purification system (Elga, High Wycombe, UK). GW660511X and omapatrilat were prepared according to the procedure given by the patent WO9724342 and by Robl *et al.* [21], respectively.

Preparation of Plasma

Human blood was supplied in-house; Han Wistar rats (Charles River) were anaesthetized (oxygen/nitrous oxide/isoflurane delivered by Ohmeda vaporizer) and whole blood was removed using a syringe by cardiac puncture through the diaphragm. Human or rat blood was transferred to heparinized sample tubes (20 U/ml ICN 101932). Plasma was obtained after centrifugation at 2000 g at room temperature for 10 min and transferred to fresh tubes and stored at −70 °C until analysis.

Incubation Procedure

The protocol used was a modified version of that described by Dendorfer *et al.* [7]. 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+}. To 1100 μl of buffer containing 4 μM of BrBK was added GW660511X or omapatrilat (27 μl); the resulting solution was mixed and then human or rat plasma (33 μl) was added to give an incubation volume of 1160 μl. At different time points 110 μl of the mixture was taken and supplemented with TFA (2.2 μl) and centrifuged at 4 °C for 30 min at 2000 g. An aliquot of the supernatant (10 μl) was injected onto the LC-MS/ICPMS system. The samples were maintained at 4 °C in the autosampler.

HPLC-UV-MS/ICPMS Analysis

The spectra of BrBK and its metabolites were determined as described previously [20]. Briefly, separation was performed on a Waters XterraMS 150 × 2.1 mm with elution (0.4 ml/min) using a linear reversed phase gradient based around 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B). The flow of the LC pump was split equally between oaTOF/MS (LCT, Micromass, Manchester) and ICPMS (Platform Life, Micromass, Manchester UK). The structures of BrBK and that of its degradation products were elucidated

by mass spectrometry and the quantification was performed by ICPMS.

Quantification Measurements of BrBK and its Metabolites

Quantification measurements were achieved by ICPMS, which is an element-specific detector with an almost uniform response independent of the structure of the metabolite [22]. Therefore, the response is only related to the molar content of the detected element. In order to use ICPMS, a synthetic form of BK was brominated in the para position of the phenylalanine referred as BrBK (Figure 3). Thus, the quantification of BrBK and its metabolites in plasma was based on the response of the detector to bromine. For all quantification studies a calibration curve produced with BrBK as a standard was linear (Figure 4) within the range of concentrations used throughout our experiments.

Data Collection and Calculations

Masslynx™ 3.4 was the software used for data acquisition and processing for both the ICPMS and oaTOF/MS instruments. The calculations and data analysis were performed on Grafit (Erithacus Software Ltd, UK). Degradation of BrBK kinetics were fitted with the mono-exponential function: $BrBK = BrBK_0.e^{-kt}$, where k is the degradation rate, and t the time. BrBK and $BrBK_0$ are the concentrations of bromobradykinin at time t and time t_0, respectively. All the data are given as mean ± SD determined in three independent experiments.

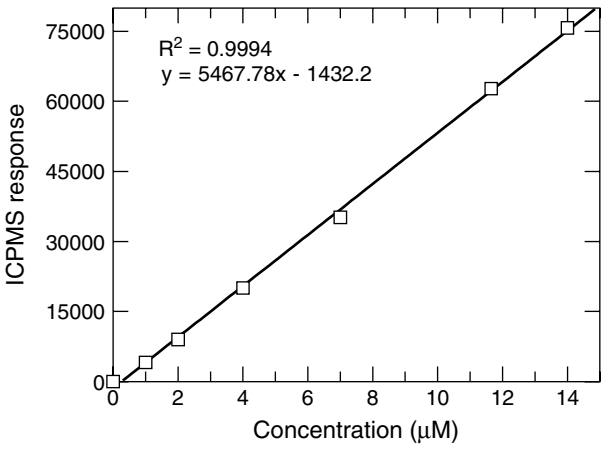

Figure 4 Calibration curve obtained with BrBK for the quantification studies.

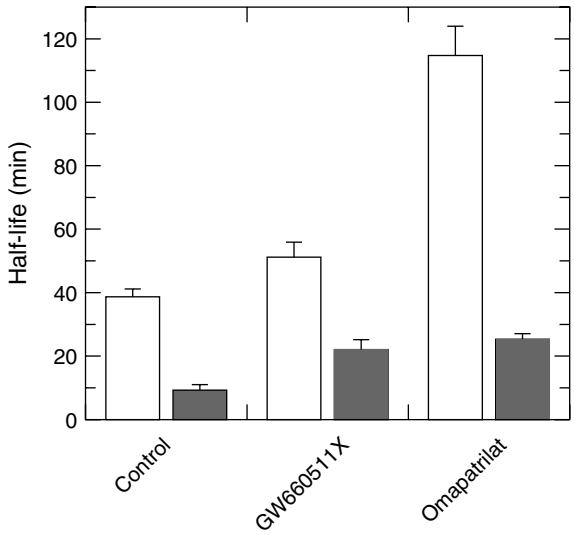

Figure 5 Half-life of BrBK after its incubation in (□) human or in (■) rat plasma in the absence of inhibitors (control) or in the presence of GW660511X or omapatrilat. The concentrations of GW660511X and omapatrilat used in human plasma studies were 3.8 μM and 32 nM, respectively. For the rat studies these concentrations were 530 nM and 50 nM.

RESULTS

Degradation of BrBK in the Presence of GW660511X or Omapatrilat

Several studies have reported the presence of ACE in both human and rat plasma [5–9]. The presence of NEP in human plasma remains controversial. As the fragment BrBK1–7 can be generated by ACE and NEP enzymes, we added 1 μM of captopril (a concentration of pure ACE inhibitor that inhibits ACE but not NEP) to rat and human plasma in order to determine the absence or presence of BrBK1–7. Under these conditions, the fragment BrBK1–7 was not detected (data not shown) thus suggesting there is no NEP in human and rat plasma. Consequently only the ACE activity of GW660511X and omapatrilat were studied in the plasma of both species. The GW660511X and omapatrilat concentrations used in the present work were equivalent to the IC_{50} values obtained in plasma with an artificial ACE substrate (HIPPURYL-His-Leu); GW660511 (3.8 μM) and omapatrilat (32 nM) in human plasma, GW660511 (530 nM) and omapatrilat (50 nM) in rat plasma

(data not shown). At these concentrations in human plasma, the half-life of BrBK (51.2 ± 4.7 min) with GW660511X and (114.7 ± 9.3 min) with omapatrilat was 2- and 3-fold higher than that obtained for the control (38.7 ± 2.4 min), respectively (Figure 5). In rat plasma, the BrBK half-life in the presence of GW660511X (22.1 ± 3.1 min) and with omapatrilat (25.3 ± 1.7 min) were similar but 3-fold higher than that obtained without inhibitor or control (9.3 ± 0.4 min) (Figure 5). Our data clearly show that there is a difference in the action of GW660511X and omapatrilat on BK metabolism between human and rat plasma.

Metabolism of BrBK in the Absence or Presence of GW660511X or Omapatrilat

The metabolism of BrBK in human plasma in the absence or in the presence of GW660511X or omapatrilat was studied at different time points over a period of 240 min and an example of an ICPMS chromatogram obtained after 120 min of incubation is shown in Figure 6. From the results reported in this figure, BrBK is degraded into BrBK1–5 and 1–7, BrBK1–8 and Br-Phe [5]. Although similar BrBK metabolites were found in the absence or the presence of inhibitors, the amount of BrBK and that of its degradation products was different as indicated by the values of the area under the peaks of each compound quoted in Figure 6.

The metabolism of BrBK in rat plasma in the absence or in the presence of GW660511X or omapatrilat was also studied at different time points over a period of 120 min and an example of ICPMS chromatogram obtained after 40 min of incubation is shown in Figure 7. From the results reported in this figure, BrBK is degraded into BrBK1–5 and BrBK1–7, BrBK1–8 and Br-Phe [5]. BrBK2–9, BrBK4–8 and BrBK2–8. Although similar BrBK

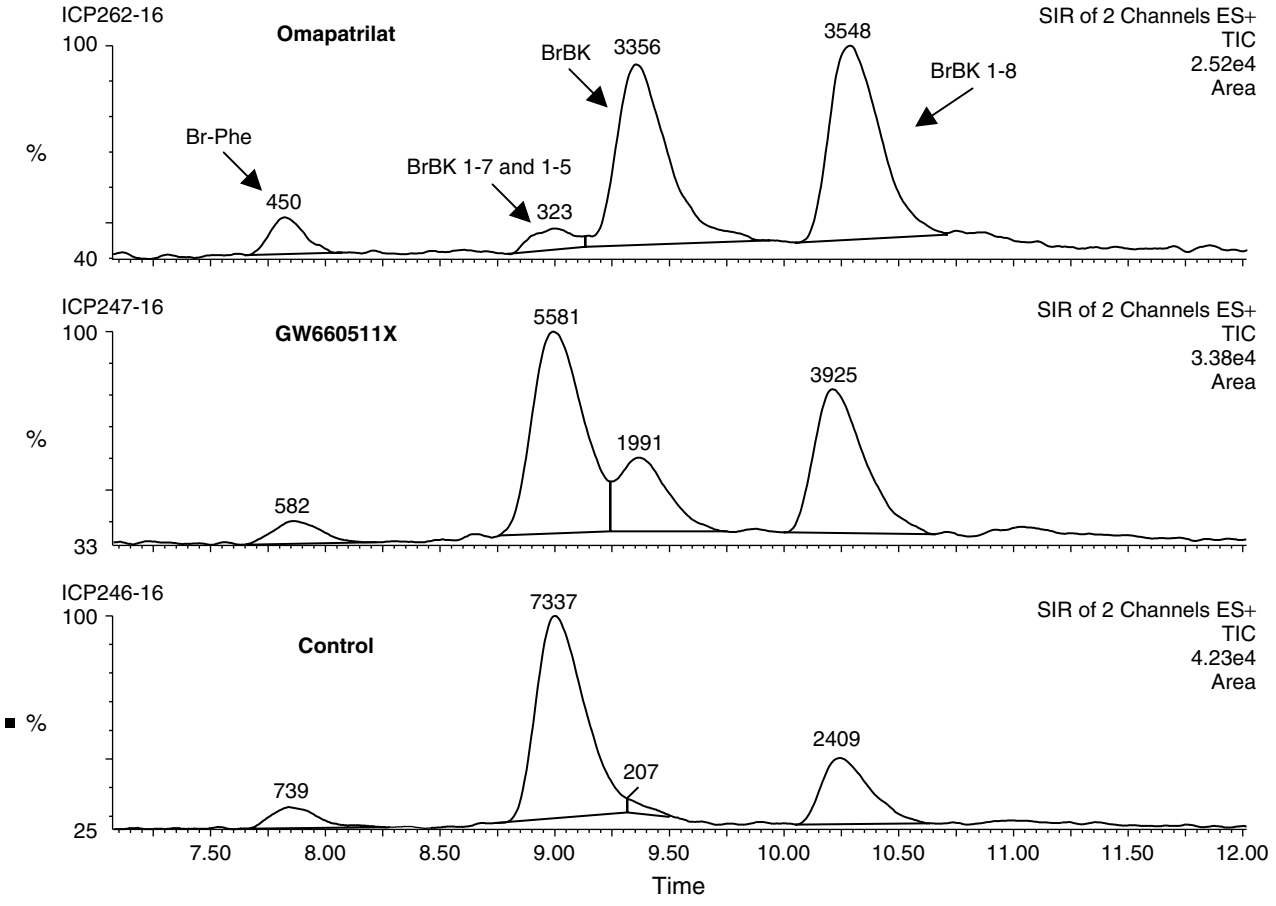

Figure 6 ICPMS chromatograms of human plasma obtained after 120 min of incubation in the absence of inhibitor (control) or in the presence of GW660511X or omapatrilat. The numbers quoted on the top of the peak represent the area under the curve.

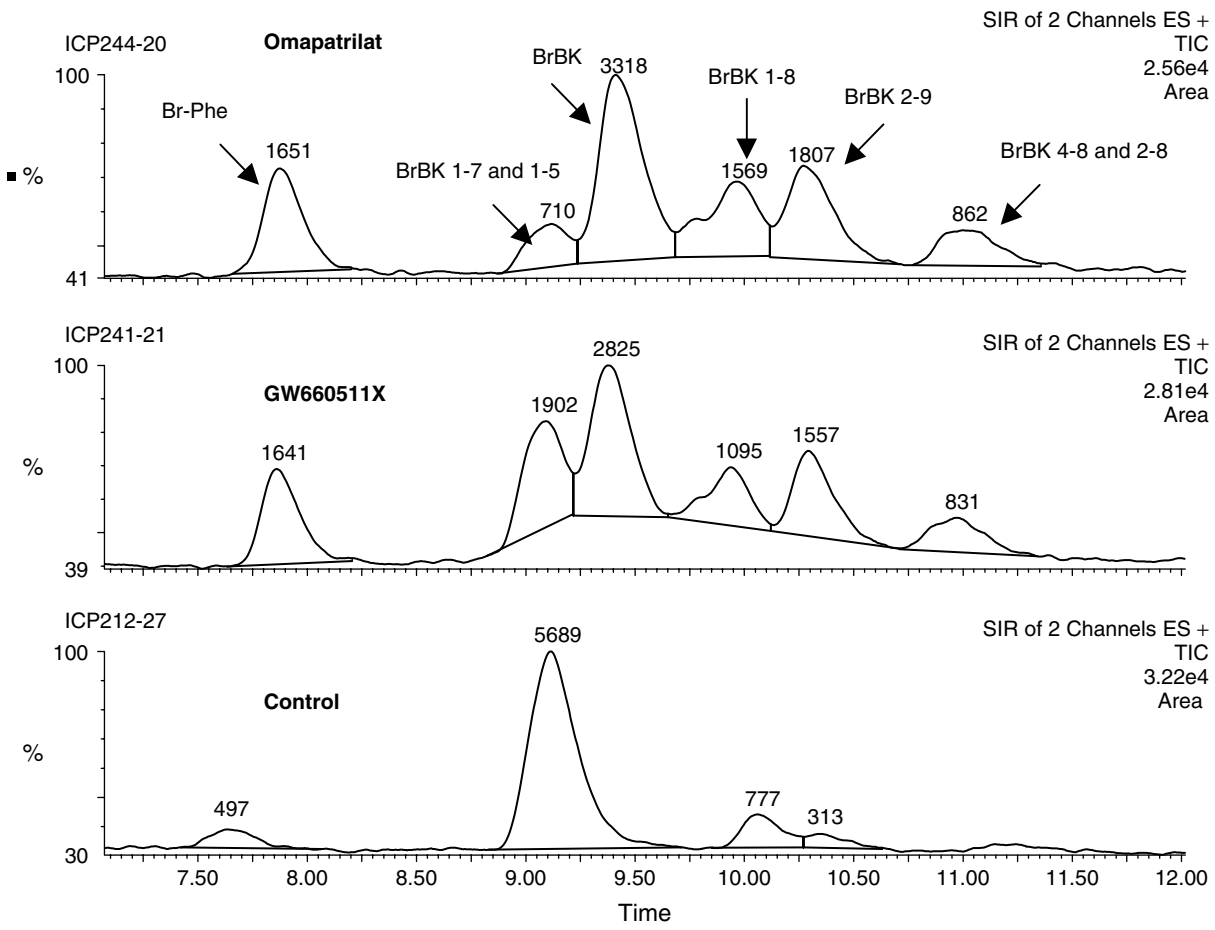

Figure 7 ICPMS chromatograms of rat plasma obtained after 40 min of incubation in the absence of inhibitor (control) or in the presence of GW660511X or omapatrilat. The numbers quoted on the top of the peak represent the area under the curve.

metabolites were found in the absence or the presence of inhibitors, the amount of BrBK and that of its degradation products was different as indicated by the values of the area under the peaks of each compound quoted in Figure 7.

Quantification of BrBK Metabolites in the Presence of GW660511X or Omapatrilat

In human plasma, omapatrilat abolished the formation of BrBK1–5 and BrBK1–7, whereas GW660511X barely decreased their formation (Figure 8A), suggesting that omapatrilat is a better ACE inhibitor than GW660511X. In addition, compared with the control, GW660511X and omapatrilat increased the concentration of the peptide BrBK1–8 with a greater effect being observed with omapatrilat (Figure 8B). This result suggests that omapatrilat and to a lesser extent GW660511X protect BrBK1–8 from degradation. Figure 9 shows the evolution of Br-Phe^5 concentration versus incubation time. It can be seen that without inhibitor Br-Phe^5 appeared 1 h after the incubation had started, suggesting that it is a secondary metabolite. Interestingly, compared with the control, GW660511X decreased the production of Br-Phe^5 whereas no change was observed with omapatrilat after 240 min of incubation.

In rat plasma, GW660511X and omapatrilat decreased the concentration of BrBK1–5 and BrBK1–7 with a greater effect being observed with omapatrilat (Figure 10A) also suggesting that omapatrilat is a better ACE inhibitor than GW660511X. However, the concentration of BrBK1–8 in both cases increased in an identical manner (Figure 10B). The peptide BrBK2–9 was only found in rat plasma and its appearance occurred in a similar manner in the presence of the two inhibitors within the first 40 min of incubation, then BrBK2–9 concentration decreased within 240 min of incubation (Figure 11A). Figure 11B

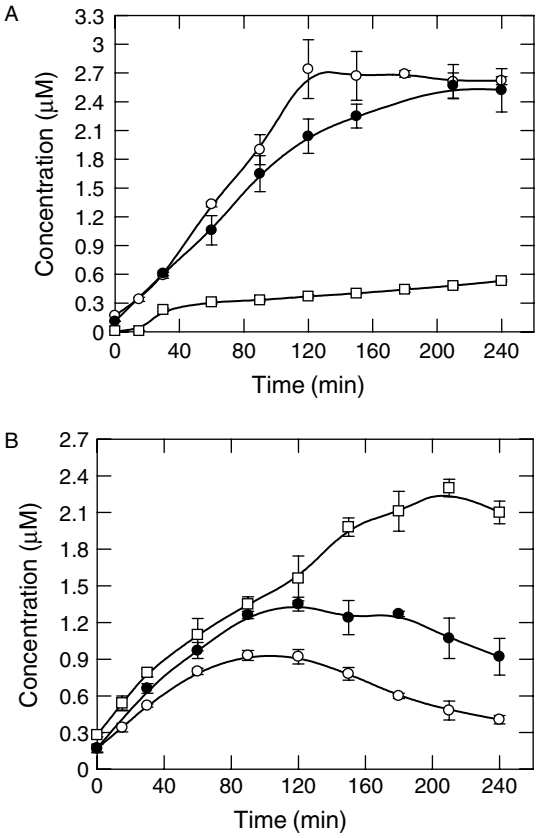

Figure 8 Evolution as a function of time of (A) BrBK1–7 and BrBK1–5, and (B) BrBK1–8 concentration in human plasma in the absence (○) no inhibitor or in the presence of (●) GW660511X (3.8 μM) or (□) omapatrilat (32 nM).

shows the evolution of Br-Phe^5 concentration. It can be noted that GW660511X and omapatrilat increased its concentration versus control, with a greater effect being observed with omapatrilat after 120 min of incubation.

DISCUSSION

The use of ACEi has been associated with the occurrence of side effects such as AE [11,12]. Accumulation of kinins namely bradykinin (BK) or its metabolites has been suggested to be involved in these effects [12–14]. The knowledge of the *in vivo* profile of two dual ACE/NEP inhibitors, which are currently undergoing clinical trials in terms of kinins (bradykinin, BK1–8 and other metabolites) accumulation, should be of great interest. Therefore, the present study deals with the investigation of the effects of two dual ACE/NEP inhibitors (GW660511X and omapatrilat) on BK

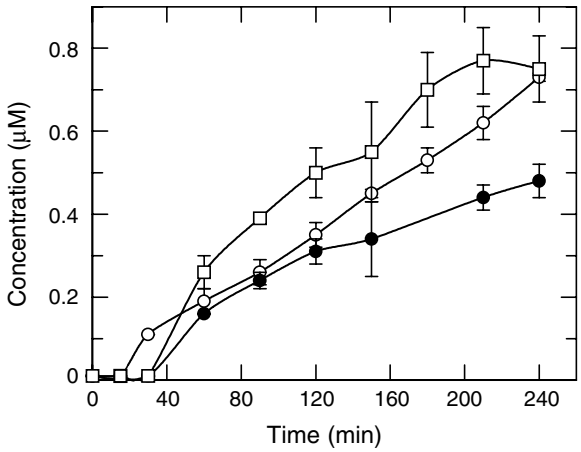

Figure 9 Evolution versus time of Br-Phe^5 concentration in human plasma in the absence (○) no inhibitor or in the presence of (●) GW660511X (3.8 μM) or (□) omapatrilat (32 nM).

metabolism and the accumulation of its degradation products in human and rat plasma by a novel approach previously described [20] and used in our laboratory. This analytical method combines HPLC-UV on-line with oaTOF-MS and ICPMS and a brominated version of BK that will allow the quantification of BK and its fragments.

In the absence of ACE/NEP inhibitors BrBK is much more rapidly degraded in rat than in human plasma. An explanation of this discrepancy could be due to the different pathways of BrBK metabolism found in rat and in human plasma. Indeed, in human plasma BrBK is primarily degraded into peptides BrBK1–8 and into BrBK1–7 and BrBK1–5 by CP N and ACE, respectively. These enzymes and also APP (which cleaves BrBK to give BrBK2–9) and DPAP (that gives peptide BrBK4–8) are present in rat plasma. Because BrBK2–9 was not found in human plasma even at a high concentration of BrBK (data not shown), it can be assumed that APP activity is absent in human plasma. This would explain some of the differences observed between rat and human plasma and is consistent with the previous *in vivo* studies performed in the rat on the physiological effect of APP [23–25]. In the present study we identified a free Phe in both human and rat plasma. Kaplan *et al.*, using HPLC with UV detection also found the latter amino acid and they suggested that its formation resulted from the cleavage of BrBK1–8 by CP B [26,27]. In our study we were able to discriminate between the two Phe of BrBK and that offers a great advantage on HPLC-UV detection. The production of Br-Phe^5 is not due to the cleavage

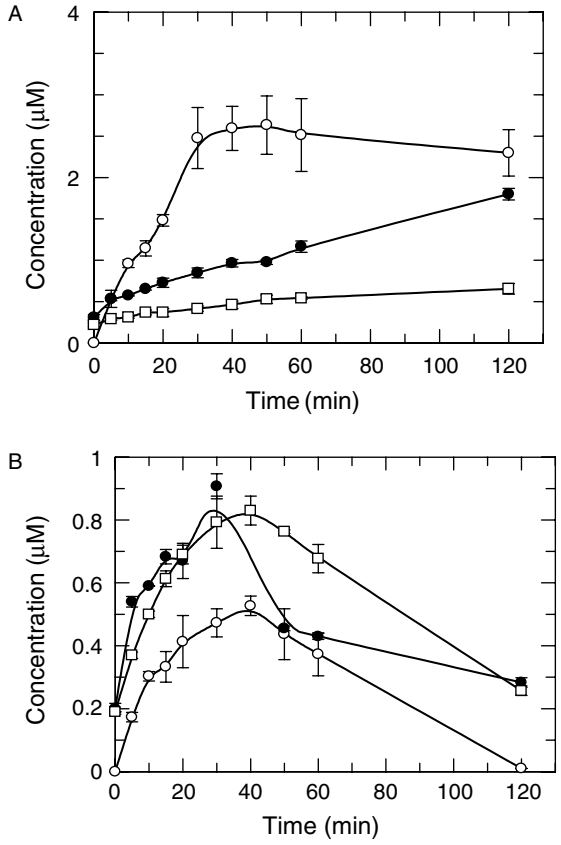

Figure 10 Evolution as a function of time of (A) BrBK1–7 and, BrBK1–5, (B) BrBK1–8 concentration in rat plasma in the absence (○) no inhibitor or in the presence of (●) GW660511X (530 nM) or (□) omapatrilat (50 nM).

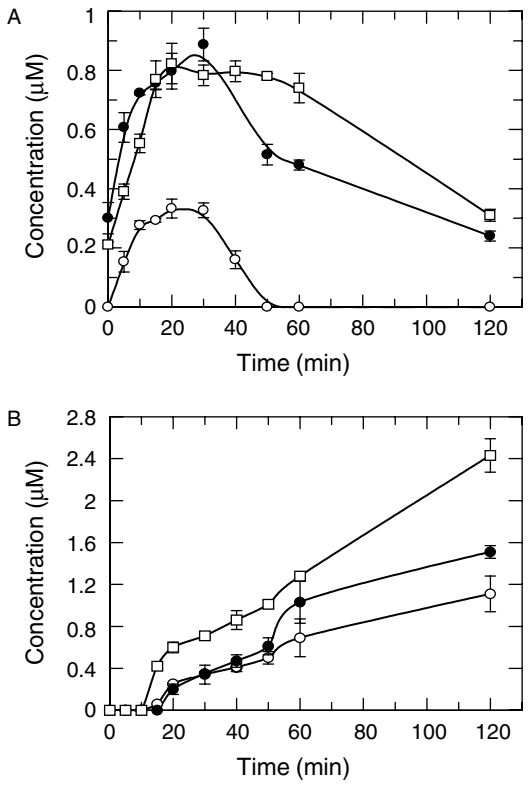

Figure 11 Evolution as a function of time of (A) BrBK2–9 and (B) Br-Phe^5 concentration in rat plasma in the absence (○) no inhibitor or in the presence of (●) GW660511X (530 nM) or (□) omapatrilat (50 nM).

of BrBK1–5 by CP N, as the latter fragment was still present in human or rat plasma samples treated with the ACE/NEP inhibitor. However, data obtained in human and rat plasma treated with the APP inhibitor (apstatin) show that the concentration of Br-Phe^5 was decreased by 3-fold compared with that of the control (data not shown). This suggests that an apstatin sensitive aminopeptidase (other than APP) could be involved in the production of Br-Phe [5].

In human plasma treated with omapatrilat the half-life of BrBK and BrBK1–8 production increase by 2- to 3-fold, respectively, in comparison with GW660511X. In addition, our data indicate that omapatrilat abolishes the production of BrBK1–5 and BrBK1–7 whereas GW660511X only partially inhibits it. Because ACE is also involved in the degradation of BrBK1–8 [28,29], the difference in ACE inhibition between GW660511X and omapatrilat could be an explanation of the discrepancy observed regarding the BrBK half-life and BrBK1–8 production. The BrBK1–8 accumulation observed with omapatrilat is of concern as regards the proposed link between BrBK1–8 level and AE [11,12]. In addition, the high production of Phe for which little is known about its toxicity in humans, would require the development of 'safer' compounds. In this respect, GW660511X, which is effective in lowering blood pressure [16,17], appears to be very interesting as our study shows little increase in BrBK1–8 production.

In rat plasma the half-life of BrBK was almost identical in the presence of omapatrilat or GW660511X. This result is in contradiction with the data obtained for human plasma where a significant increase of BrBK half-life was observed with omapatrilat compared with GW660511X. The consequence of this is that there may be some important implications for the usage of rats as *in vivo* models of hypertension. The production of BrBK1–5 and BrBK1–7 generated by ACE was inhibited in the presence of omapatrilat or GW660511X. However, a slightly better inhibition

was observed with omapatrilat. Our results indicate a difference in ACE inhibition by GW660511X between human and rat plasma. Recently, Vazeux *et al.* [30], using RXP407, a potent N-selective inhibitor of ACE, found that this compound was able to inhibit human ACE but not the rat one, suggesting a difference between the human and rat ACE *N*-domain. A separate study indicated that GW660511X preferentially interacts with the *C*-domain of human ACE (manuscript in preparation) and the implications of this domain selectivity are currently under investigation. GW660511X and omapatrilat also increased BrBK2–9 concentration. However, no evidence of the cleavage BrBK2–9 by ACE was observed *in vitro* (data not shown). Therefore the increase of BrBK2–9 concentration could be due to the potentiation of APP by ACE inhibitors as reported by Kitamura *et al.* [23].

In conclusion, our study shows that omapatrilat, which is a better ACE inhibitor than GW660511X, protects BrBK from degradation and increases the production of BrBK1–8 in both human and rat plasma, whereas GW660511X depicts a different profile of activity between human and rat plasma. Because of these differences, the rat may not be a suitable *in vivo* model for the evaluation of ACE/NEP inhibition in relation to effects in humans.

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